DEVELOPMENT OF NEW BIOSYSTEMS AND BIOCATALYSTS FOR WASTE UPGRADING INTO THE HIGH ADDED VALUE PRODUCTS BIOETHANOL AND LACTIC ACID

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A mio cugino

“Somewhere, something incredible is waiting to be known”

Carl Sagan

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Abstract

Valorization of lignocellulosic biomass components leads to several possibilities for the production of valuable chemicals, fuels and energy that could be used by modern societies in substitution to conventional ones derived from fossil-based resources. This work was mainly aimed at enlarging the assortment of biosystems and biocatalists (microbes and/or enzymes) for generation of high added value bio-products, such as sugars, ethanol and lactic acid, from both dedicated crops and lignocellulosic wastes. Particularly, **32 different fungal strains**, belonging to the collection of the Department of Engenharia de Bioprocessos e Biotecnologia (University Federal do Paraná, Brasil) (DEBB), were investigated for ligninolytic, cellulolytic and xylanolytic activities production on solid and liquid media. The screening led to the selection of ***Aspergillus niger*** **NRRL 3312**, as the best cellulase and xylanase activities producer and the strain ***Pleurotus sajor-caju*** **INRA 3501** for the laccase activity production. **Brewers' spent grain** (BSG) was shown a suitable substrate for the low cost lignocellulolytic enzymes production by solid state fermentation with the selected strains; indeed, *A. niger* NRRL 3312 reached a maximum cellulase and xylanase activities production of 118.04 U $gram^{-1}$ of dry substrate (gds) and 1315.15 U gds^{-1}, respectively, wilst *P. sajor-caju* INRA 3501 showed a maximum laccase production of 157.36 U gds^{-1}. By proteomic analyses peptides belonging to the enzymes responsible for the cellulase and xylanase activities from *A. niger* NRRL 3312 and for the laccase acitivity from *P. sajor-caju* INRA 3501 were identified. **An enzymatic mixture of cellulases and xylanases**, produced by the strain ***Pleurotus ostreatus*** using microcrystalline cellulose as inducer, was partially characterized and tested in the statistical analysis of *Arundo donax* saccharification. The Plackett-Burman screening design, applied to identify the most significant parameters for the maximum sugars release, showed that the most significant influence was exercised by temperature, pH, and time. The combined effect of these factors on the saccharification process was analyzed by a 3^3 factorial experimental design and the best result of 480.10 mg of sugars gds^{-1} was obtained at 45 °C, pH 3.5, and 96 hours of incubation. **Alkaline-acid (AAT) pretreated BSG** was evaluated for ethanol production after enzymatic hydrolysis with commercial enzymes. The obtained hydrolysate, with a glucose concentrationn of 75 g/L, diluted to 50 g/L, was used for fermentation by the strain ***Saccharomyces cerevisiae*** **NRRL YB 2293**, selected as the best producer among five ethanologenic microorganims belonging to the DEBB collection. When the hydrolysate was supplemented with yeast extract, 12.79 g/L of ethanol, corresponding to 55 % of the maximum theoretical value, was obtained within 24 hours, while in the non-supplemented hydrolysate, a similar concentration was reached within 48 hours; indeed, the added nitrogen source reduced the ethanol fermentation time and promoted glucose uptake and cell growth. ***Lactobacillus acidophilus*** **ATCC 43121**, selected as the best lactic acid producer among six *Lactobacillus* strains belonging to the DEBB collection, was analyzed for its ability to grow on the hydrolysates obtained from **BSG after AAT or aqueous ammonia soaking (AAS) pretreatment**. The lactic acid production by *L. acidophilus* ATCC 43121 through fermentation of the hydrolysate from AAS treated BSG was 96 % higher than that from the AAT treated one, although similar yields of lactic acid per consumed glucose were achieved. The best results were obtained by fermentation of AAS BSG hydrolysate supplemented with yeast extract, in which the strain produced 22.16 g/L of lactic acid (yield of 0.61 g/g), 27 % higher than the value (17.49 g/L) obtained in the absence of a nitrogen source.

Le biomasse lignocellulosiche: l'alternativa alle risorse fossili non rinnovabili

Nel 2014, secondo una stima dell'International Energy Agency (IEA), la richiesta energetica mondiale è stata soddisfatta principalmente mediante l'impiego di risorse fossili, quali petrolio (39.9 %), carbone (11.5 %) e gas naturali (15.1 %) [1]. E' stato inoltre stimato che petrolio, carbone e gas naturali si esauriranno in circa 35, 107 e 37 anni, rispettivamente, con la conseguenza che il carbone rappresenterà l'unica fonte di energia disponibile dopo il 2042 [2].

La limitazione delle energie fossili, insieme al surriscaldamento globale, alle elevate emission di gas ad effetto serra, all'incessante crescita demografica ed alle problematiche legate al costo dello smaltimento dei rifiuti, sta muovendo l'interesse mondiale verso lo sviluppo di un'economia basata su fonti di energia rinnovabili. La Direttiva Europea 2009/28/CE, sulla promozione della produzione e dell'uso dei biocombustibili, ha stabilito che questi dovranno sostituire del 20 % i carburanti fossili (benzina e diesel) entro il 2020, fissando per tutti gli Stati membri un obiettivo del 10 % per la quota di energia da fonti rinnovabili nei trasporti.

In quest'ottica di sviluppo di un'economia sostenibile che possa minimizzare le problematiche delle moderne società industriali, l'attenzione è stata rivolta alla valorizzazione delle biomasse lignocellulosiche. Esse consistono principalmente in colture dedicate e residui agro-industriali e data la loro abbondanza e composizione, equa distribuzione geografica e basso costo sono, considerate tra le più promettenti fonti rinnovabili in alternativa a quelle fossili [3]. La coltivazione su larga scala delle colture dedicate, come la pianta perenne *Arundo donax*, su terreni marginali e/o contaminati, si sta diffondendo in tutto il mondo minimizzando, in questo modo, il dislocamento delle colture a destinazione alimentare, favorendo inoltre, la fertilità dei suoli impiegati [4]. D'altro canto, l'utilizzo dei residui agro-industriali consente di ridurre ulteriormente il conflitto food *versus* fuel, oltre che rispondere alle problematiche connesse alla deforestazione [5].

Le biomasse lignocellulosiche sono costituite principalmente da tre polimeri – cellulosa, emicellulosa e lignina - organizzati in una complessa ed amorfa struttura tridimensionale, con una composizione relativa varabile a seconda della specie considerata. La cellulosa, composta da unità di D-glucosio legate attraverso legami β-1,4 glicosidici, rappresenta la componente maggioritaria delle biomasse, con una percentuale di circa il 40-50 % del peso secco totale, ed è incastonata in una matrice di emicellulosa e lignina. L'emicellulosa è il secondo polimero più abbondante con circa il 25-35 % del peso secco totale ed è costituita da diversi monosaccaridi, come xilosio, arabinosio, mannosio e galattosio, organizzati a formare un polimero eterogeneo e ramificato. La lignina, invece, rappresenta la componente minoritaria delle biomasse lignocellulosiche (10-25 % del peso secco totale) e consiste principalmente di composti aromatici legati mediante legami esteri [6].

Dato l'elevato contenuto in polisaccaridi, le biomasse lignocellulosiche possono essere convertite in un ampio ventaglio di prodotti d'interesse industriale, quali carburanti, polimeri, enzimi, additivi alimentari, coloranti, composti aromatici ed acidi organici mediante lo sviluppo e l'ottimizzazione di processi biotecnologici che siano eco-compatibili e che possano rispondere alle necessità energetiche mondiali.

Processo di conversione delle biomasse lignocellulosiche in prodotti ad alto valore aggiunto

La valorizzazione delle biomasse lignocellulosiche richiede la conversione della componente (emi)cellulosica in zuccheri fermentabili, previa rimozione della lignina, che conferisce grande resistenza meccanica e rende inaccessibili le componenti polisaccaridiche. Il processo convenzionale attualmente adottato prevede un iniziale pretrattamento per rimuovere la componente ligninica, seguito da una fase di idrolisi della componente polisaccaridica mediante metodi chimici o enzimatici, ed infine, una fase di fermentazione degli zuccheri pentosi ed esosi.

Un pretrattamento per essere efficiente deve aumentare l'accessibilità e la digeribilità della frazione (emi)cellulosica al fine di migliorare la successiva fase di idrolisi. Molti studi di conversione si sono focalizzati sull'ottimizzazione della fase di pretrattamento al fine di massimizzare la rimozione di lignina ed, allo stesso tempo, minimizzare la perdita di zuccheri, la formazione di composti tossici ed i costi del processo. Il pretrattamento, attualmente, può essere condotto mediante metodi fisici, chimici e biologici. Sebbene i metodi chimico-fisici siano maggiormente impiegati rispetto a quelli biologici data l'elevata resa di delignificazione, non sono considerati eco-compatibili a causa delle elevate energie richieste e dell'utilizzo di costose sostanze chimiche; inoltre uno svantaggio di tali trattamenti è la generazione di composti tossici che interferendo sia con le attività enzimatiche nella fase di idrolisi che con la crescita microbica nella fase di fermentazione, richiedono trattamenti aggiuntivi volti alla detossificazione della biomassa, con conseguente aumento di reflui e scarti, oltre che dei costi del processo [7].

Di conseguenza, il pretrattamento biologico, basato sull'applicazione di microrganismi o enzimi, è considerato un processo di delignificazione promettente, essendo eco-compatibile ed economico [8]. In natura, una grande varietà di microrganismi, sia batteri che funghi, è in grado di produrre alti livelli di enzimi ligninolitici, volti alla rimozione della lignina e di enzimi (emi)cellulolitici per la conversione della componente (emi)cellulosica in zuccheri fermentabili [9]. Principalmente, il pretrattamento biologico viene effettuato mediante fermentazione su stato solido di funghi e/o batteri, mimando il più possibile le condizioni che si verificano in natura, quali basse temperature e pressioni, minime quantità o totale assenza di acqua libera; inoltre, non sono richiesti composti chimici e la produzione di reflui e di sostanze tossiche è estremamente ridotta [10]. Ciononostante, il processo non trova ampia applicazione a causa dei lunghi tempi richiesti (3-5 mesi) per la delignificazione, responsabile della perdita di un'elevata percentuale di polisaccaridi e la conseguente diminuzione delle rese nella fase di idrolisi.

La delignificazione mediante enzimi - lignina perossidasi, laccasi e manganese perossidasi - rappresenta una valida alternativa biologica all'utilizzo di funghi e batteri, garantendo alte rese di delignificazione in tempi brevi, senza richiesta di nutrienti nè consumo di polisaccaridi. La principale limitazione di tale processo consiste nell'elevato costo di produzione degli enzimi ligninolitici che ne impedisce l'applicazione su larga scala [11].

Una volta rimossa la lignina, l'idrolisi della cellulosa è realizzata mediante trattamenti chimici o enzimatici. Questi ultimi sono basati sull'azione cooperativa di tre classi di enzimi: endoglucanasi, cellobioidrolasi e β-glucosidasi. Inoltre al fine di aumentare le rese di idrolisi, ed ottenere zuccheri fermentabili non solo dalla componente cellulosica ma anche da quella emicellulosica, il processo prevede l'impiego di miscele enzimatiche costituite non solo da cellulasi ma anche emicellulasi, quali xilanasi, mannanasi, pectinasi e tutti gli altri enzimi che agiscono sulle catene laterali

di xilani e mannani. Ciò richiede l'impiego di miscele enzimatiche costituite non solo da cellulasi ma anche emicellulasi, quali xilanasi, mannanasi, pectinasi e da tutti gli altri enzimi che agiscono sulle catene laterali di xilani e mannani.
Il processo di idrolisi rappresenta la principale sfida da superare per rendere competitivo ed applicabile su scala industriale il processo di bioconversione delle biomasse lignocellulosiche, dato l'elevato costo degli enzimi. Infatti, una recente valutazione tecno-economica attribuisce circa il 20 % del costo dell'intero processo di bioconversione proprio alla produzione di enzimi (emi)cellulolitici, coinvolti nell'idrolisi delle componenti macromolecolari per l'ottenimento di zuccheri fermentabili.
La fase finale dell'intero processo richiede la fermentazione microbica degli zuccheri pentosi ed esosi in prodotti ad alto valore aggiunto. Il processo di fermentazione può essere condotto separatamente dall'idrolisi (Separate Hydrolysis and Fermentation) o in maniera simultanea (Simultaneous Saccharification and Fermentation) [12]. Una strategia più innovativa e che potrebbe ridurre di molto i costi del processo è poi rappresentata dal Consolidated BioProcessing (CBP), che si basa sull'impiego di un singolo microorganismo, ingegnerizzato a produrre un cocktail enzimatico, che consenta la diretta conversione della cellulosa in prodotti di interesse commerciale.

Bioetanolo di seconda generazione

Il bioetanolo di seconda generazione è considerato, tra tutti i biocombustibili, un'ottima alternativa ai carburanti fossili apportando una serie di vantaggi, quali la significativa riduzione di emissioni di CO_2, particolato, NO_x e SO_x [13]. Nonostante l'utilizzo del bioetanolo in sostituzione dei carburanti fossili si sia diffuso fin dagli inizi del 1900 in Brasile, USA ed Europa, a causa degli elevati costi di produzione le sue potenzialità sono state sottovalutate fino al 1970, quando la crisi energetica ha mosso l'interesse mondiale verso l'uso di risorse rinnovabili [14]. Nell'ultimo decennio, molti Paesi hanno emesso diversi "Fuel Ethanol Programs", volti a rendere obbligatorio l'uso di miscele di etanolo attraverso incentivi o esenzioni fiscali, determinando così un rapido incremento della produzione di bioetanolo [15]; ciò ha contribuito a diminuire la dipendenza dai carburanti importati, incentivando l'economia rurale.
Nel 2014, la produzione globale di bioetanolo ha raggiunto il record di 14,3 miliardi di galloni, eclissando il precedente record di 13.9 miliardi di galloni risalenti al 2011. Il Paese leader nella produzione di bioetanolo è rappresentato dagli USA con circa il 60 % della produzione mondiale; il Brasile, invece, con circa 6.2 miliardi di galloni è responsabile del 25 % della produzione mondiale, seguito dall'Europa con circa il 6 % [16].
Sulla base della materia prima utilizzata si distinguono un etanolo di "prima generazione" ed un etanolo di "seconda generazione". Il primo è prodotto a partire da materie edibili, quali grano o semi, mediante un semplice processo caratterizzato da estrazione degli zuccheri, fermentazione e distillazione. Per la produzione di etanolo di prima generazione, il Brasile utilizza principalmente la canna da zucchero, mentre gli USA e l'Europa utilizzano amido estratto dal mais e, dal frumento e dall' orzo, rispettivamente. Ma l'utilizzo di materie edibili per la produzione di etanolo di prima generazione trova dei limiti nell'insufficienza di tali risorse nonché nella loro destinazione ad uso alimentare [17].
Per superare questi ostacoli, l'interesse della comunità scientifica si è rivolto allo sviluppo di etanolo di seconda generazione, prodotto a partire da biomasse lignocellulosiche, note per essere non edibili, rinnovabili ed ampiamente disponibili in

tutto il Mondo. Ciò ha consentito di evitare l'impiego di colture a destinazione alimentare e di conseguenza, il cosiddetto conflitto "food *versus* fuel".

Acido lattico

L'acido lattico è un acido organico riconosciuto come composto GRAS (Generally Recognized As Safe) dalla Food and Drug Administration (US FDA) e di conseguenza trova larga applicazione nell'industria alimentare, cosmetica, farmaceutica e chimica [18]. Circa il 70 % di acido lattico prodotto è utilizzato nell'industria alimentare per la produzione di formaggi e yogurt [19]. Nell'industria cosmetica, l'acido lattico è utilizzato in prodotti destinati all'igiene ed all'estetica, grazie agli effetti antimicrobici e rigeneranti riscontrati sulla pelle [20]. Inoltre, nell'industria farmaceutica e chimica, l'acido lattico è utilizzato come precursore di piccoli e grandi polimeri, quali acido polilattico e polimeri acrilici, che vengono impiegati nella produzione di imballaggi, protesi, suture e dispositivi per il dosaggio dei farmaci essendo biocompatibili e biodegradabili [21].
E' stato calcolato che nel 2007 la richiesta globale di acido lattico è stata di circa 130.000-150.000 tonnellate, con un valore commerciale variabile tra 1.38 US$ kg^{-1} e 1.54 US$ kg^{-1} in dipendenza del grado di purezza [22]. E' previsto che la richiesta di acido lattico aumenti notevolmente nei prossimi anni, quindi è di interesse globale lo sviluppo di un processo economico ed efficiente che garantisca elevate rese di produzione. In particolare, l'attenzione è rivolta alla messa appunto di fermentazioni microbiche basate sull'utilizzo di substrati a basso costo come, ad esempio, idrolizzati ottenuti dal pretrattamento e saccarificazione di biomasse lignocellulosiche [23,24,25].

Strategie per l'incremento della competitività del processo di conversione delle biomasse lignocellulosiche in prodotti ad alto valore aggiunto

Numerosi progressi sono tutt'ora necessari per lo sviluppo di processi eco-compatibili e competitivi che consentano la conversione delle biomasse lignocellulosiche in prodotti di interesse commerciale.
Le principali problematiche riscontrate nella fase di pretrattamento sono la bassa riproducibilità del processo (che dipende dall'equilibrio di numerose variabili, quali pH e temperatura), la sterilità e scalabilità a livello industriale. Al fine di ovviare tali limitazioni, molte ricerche sono focalizzate i) all'isolamento ed identificazione di nuovi microrganismi ligninolitici e/o all'impiego di co-culture microbiche che possano essere più produttive anche in condizioni di stress [26]; ii) all'identificazione, tramite l'utilizzo di software statistici, di una combinazione di parametri operativi ottimale che garantisca alte rese [27], ed iii) alla progettazione di nuove configurazioni bioreattoristiche che consentano l'applicazione su scala industriale del processo di fermentazione su stato solido [28].
Per quanto concerne la fase di idrolisi, l'obiettivo principale è quello di ridurre i costi di produzione degli enzimi. Una strategia impiegata è quella di utilizzare substrati a basso costo, come le biomasse lignocellulosiche, per la coltivazione tramite fermentazione su stato solido di ceppi fungini noti per essere buoni produttori di enzimi (emi)cellulolitici. Ciononostante, in molti casi, tali enzimi non risultano possedere delle performance ottimali, dovute alle basse specificità per il substrato e al limitato range di pH e temperatura ottimale per l'attività idrolitica. Ciò ha spinto la comunità scientifica alla ricerca di enzimi dalle prestazioni migliorate. Grazie alla combinazione di diversi approcci, quali genomico, trascrittomico e proteomico, è possibile i) migliorare gli enzimi mediante strategie di mutazione sito-diretta o di

evoluzione guidata [29]; ii) identificare nuovi enzimi con performances migliorate mediante approccio metagenomico [30]; iii) sviluppare ceppi microbici ingegnerizzati per produrre alti livelli di enzimi mediante sistemi di espressione ottimizzati [31].
L'incremento delle rese del processo di fermentazione, invece, è auspicabile mediante l'utilizzo di microrganismi che abbiano efficienti sistemi di internalizzazione e trasporto degli zuccheri, che siano in grado di fermentare sia esosi che pentosi e che mostrino elevata tolleranza ai prodotti intermedi e/o finali di fermentazione. La selezione e caratterizzazione di nuovi microrganismi, provenienti da differenti habitat, può consentire l'identificazione di ceppi in grado di rispondere a queste esigenze. Inoltre, l'ingegneria genetica consente di sviluppare ceppi microbici dalle perfomances migliorate utilizzabili su scala industriale [32].
Questo progetto di dottorato fornisce avanzamenti nel settore della valorizzazione delle biomasse lignocellulosiche, attraverso l'identificazione di nuovi biosistemi e biocatalizzatori adoperabili, in particolar modo, nei processi di conversione per la produzione di bioetanolo ed acido lattico.

Preparazione delle biomasse lignocellulosiche da utilizzare nei processi di bioconversione

Nell'ambito del progetto BIOASSORT (318931_Improvement of technologies and tools, e.g. biosystems and biocatalysts, for waste conversion to develop an assortment of high added value eco-friendly and cost-effective bio-products; Marie Curie Action IRSES 2012), questo progetto di dottorato ha previsto la conversione dallo scarto ottenuto dalla produzione della birra (BSG), costituito da bucce di grani esausti dopo macerazione e chiarificazione, in prodotti ad alto valore aggiunto. Al fine di ottenere un idrolizzato ricco in zuccheri fermentabili da convertire in acido lattico ed etanolo di seconda generazione, lo scarto BSG è stato sottoposto a pretrattamenti chimico-fisici, volti a rimuovere la componente ligninica, ed a saccarificazione mediante mix enzimatiche commerciali. Lo scarto BSG utilizzato in questo progetto di dottorato è stato gentilmente offerto dal birrificio Bier Hoff (Curitiba-PR, Brasile) (BSG1) e dal birrificio Maneba Striano (Napoli, Italia) (BSG2).

- **Pretrattamento acido-alcalino e saccarificazione dello scarto BSG**

Lo scarto BSG1 è stato sottoposto a pretrattamento acido-alcalino, utilizzando acido solforico per solubilizzare la componente emicellulosica e la soda per rimuovere la componente ligninica. Il pretrattamento ha consentito la rimozione dell'88.68 %, 41.22 % e 84.54 % della frazione emicellulosica, ligninica e di altre componenti - polveri, proteine ed estrattivi - rispettivamente, generando un residuo solido con un contenuto in cellulosa pari all'86.49 %. Quest'ultimo è stato saccarificato mediante una mix di enzimi commerciali, con un'efficienza di conversione della cellulosa in glucosio pari al 97 %. L' idrolizzato così ottenuto, indicato come AAT BSG1 e caratterizzato da una concentrazione di glucosio pari a 75 g/L, è stato utilizzato come substrato per la produzione di acido lattico ed etanolo di seconda generazione.

- **Trattamento Aqueous Ammonia Soaking e saccarificazione dello scarto BSG**

Lo scarto BSG2 è stato sottoposto al pretrattamento Aqueous Ammonia Soaking (AAS) che ha consentito la solubilizzazione di una cospicua percentuale di lignina (62.26 %), preservando la componente emicellulosica e generando un residuo solido con un contenuto in cellulosa ed emicellulosa del 57.09 % e 34.89 %, rispettivamente. Infatti, come riportato in letteratura, l'AAS è un metodo che

incrementa la digeribilità della biomassa attraverso la rimozione della lignina, senza però degradare la frazione (emi)cellulosica e generare prodotti secondari che possano inibire le successive fasi di idrolisi e fermentazione.
Il residuo solido è stato poi saccarificato mediante una mix di enzimi commerciali, raggiungendo una resa di conversione della cellulosa in glucosio del 86.8 %. L'idrolizzato AAS BSG2, con una concentrazione di glucosio pari a 60 g/L, è stato utilizzato come substrato per la produzione di acido lattico.

Sviluppo di nuovi biosistemi e biocatalizzatori per la valorizzazione di biomasse lignocellulosiche

Nell'ambito del progetto BIOASSORT, al fine di ampliare l'assortimento di biosistemi da utilizzare nei processi di conversione di biomasse lignocellulosiche in prodotti ad alto valore aggiunto, è stato condotto uno screening per la selezione di microrganismi lignocellulolitici, etanologenici e lattici dalla collezione "Strain Collection of the Bioprocess and Biotechnology Division" (DEBB) del partner brasiliano BIOASSORT (Dipartimento di "Engenharia de Bioprocessos e Biotecnologia") presso l'Università Federal do Paraná (Curitiba-PR, Brasile).
Inoltre, nell'ambito del progetto Biopolis ("Development of Green Technologies for Production of Biochemicals and Their Use in Preparation and Industrial Application of Polimeric Materials from Agricultural Biomasses Cultivated in a Sustainable Way in Campania Region"_ PON03PE 00107 1), il fungo basidiomicete *Pleurotus ostreatus* è stato utilizzato come fonte di nuovi biocatalizzatori, quali enzimi cellulolitici e xilanolitici, da utilizzare per l'idrolisi delle biomasse lignocellulosiche.

- **Selezione e caratterizzazione di nuovi microrganismi (emi)cellulolitici e ligninolitici e loro enzimi**

32 ceppi fungini, appartenenti al genere *Lentinus*, *Aspergillus* e *Pleurotus*, della collezione DEBB sono stati sottoposti a screening funzionale per la produzione di attività cellulasica e xilanasica usando un terreno di coltura solido contenente come unica fonte di carbonio la carbossimetilcellulosa (CMC) e lo xilano, rispettivamente. La selezione è stata condotta calcolando il valore dell'Enzymatic Index (EI), dato dal rapporto tra la dimensione dell'alone di attività, rilevato attraverso colorazione con Congo Red, e la dimensione del micelio fungino. Sono stati selezionati i microrganismi con un EI uguale o superiore al valore di 1.4. Lo screening per l'attività cellulasica ha permesso di selezionare i ceppi *Lentinus edodes* INRA L 465, *L. edodes* INRA 790 e *L. edodes* INRA 1022, mentre i ceppi *L. edodes* INRA 6635, *L. edodes* INRA 6647, *L. edodes* INRA 6614, *Aspergillus niger* NRRL 599, *A. niger* NRRL 2270, *A. niger* NRRL 334, *A. niger* NRRL 3312, *A. niger* NRRL 1278 e *A. niger* NRRL 511 sono stati selezionati sia per attività cellulasica che xilanasica.
Tutti i ceppi fungini sono stati analizzati anche per la loro abilità di produrre enzimi ligninolitici mediante screening su terreno solido contenente l'indicatore colorimetrico guaiacolo. Attraverso la misurazione dell'alone di attività, dovuto all'ossidazione del guaiacolo ad opera degli enzimi ligninolitici, è stato possibile selezionare i ceppi *L. edodes* INRA 797, *L. edodes* INRA 6608, *L. edodes* INRA 6646, *Pleurotus sajor-caju* INRA 31, *P. sajor-caju* INRA 3501 e *P. sajor-caju* INRA 3824.
I ceppi selezionati mediante screening su mezzo solido sono stati sottoposti ad analisi quantitativa per valutare la loro abilità di produrre enzimi cellulolitici, xilanolitici e ligninolitici in coltura liquida.
I risultati dell'analisi hanno permesso di selezionare 3 ceppi. Il ceppo *A. niger* NRRL 3312 come il massimo produttore sia di attività cellulasica (0.28 U/mL) che xilanasica

(19.18 U/mL), il ceppo *L. edodes* INRA L 465 per l'alta attività cellulasica (0.23 U/mL) e il ceppo *P. sajur-caju* INRA 31 come il miglior produttore di attività laccasica (12.07 U/mL).
I 3 ceppi fungini selezionati mediante screening qualitativo e quantitativo sono stati ulteriormente testati per la loro abilità nel produrre le attività enzimatiche di interesse mediante fermentazione su stato solido sugli scarti della produzione della birra (BSG), al fine di ottenere mix enzimatiche a basso costo da utilizzare nell'idrolisi delle biomasse lignocellulosiche.
A. niger NRRL 3312 è risultato efficiente nel colonizzare lo scarto BSG, raggiungendo un picco di produzione di cellulasi pari a 118.04 U g^{-1} di sostanza secca (gds^{-1}) ed un valore massimo di xilanasi di 1315.15 U gds^{-1} al 10° e 4° giorno di fermentazione, rispettivamente.
Anche il ceppo *P. sajor-caju* INRA 3501 è risultato capace di colonizzare lo scarto BSG, raggiungendo la massima produzione di laccasi pari a 157.36 gds^{-1} al 4° giorno di fermentazione.
Per quel che concerne il ceppo *L. edodes* INRA L 465, non sono stati condotti ulteriori esperimenti in quanto esso non ha dimostrato capacità di colonizzazione dello scarto BSG.
Attraverso analisi di zimografia accoppiate ad analisi di proteomica è stato possibile identificare le attività d'interesse prodotte dai due microrganismi selezionati.

- **Selezione di nuovi microrganismi etanologenici per la produzione di etanolo di seconda generazione**

Al fine di selezionare un microrganismo etanologenico da utilizzare nei processi di conversione delle biomasse lignocellulosiche in etanolo di seconda generazione, sei ceppi - *Saccharomyces cerevisiae* NRRLY 12,908, *S. cerevisiae* NRRL YB 2293, *S. cerevisiae* NRRL Y 11,878, *S. cerevisiae* NRRL Y 2034 e *Zigosaccharomyces rouxi* NRRL Y 2547 - appartenenti alla collezione DEBB, sono stati analizzati per la loro abilità a produrre etanolo in mezzo sintetico. Dopo 72 ore di fermentazione a 30 °C, è stato misurata la concentrazione di etanolo, ottenendo valori variabili tra 9.01 ± 0.05 g/L e 10.82 ± 0.03 g/L. Il ceppo *S. cerevisiae* NRRL YB 2293 ha mostrato la maggiore resa pari a 0.27 g di etanolo su g di glucosio, corrispondenti al 53 % della massima resa teorica. Tale ceppo è stato selezionato per la conversione dall'idrolizzato AAT BSG1 in etanolo di seconda generazione.

- **Selezione di nuovi microorganismi per la produzione di acido lattico**

Sei ceppi - *L. acidophilus* ATCC 53672, *L. acidophilus* ATCC 43121, *L. acidophilus* ATCC 4356, *L. lactis* INRA 18, *L. pentosus* NRRL B-227, e *L. plantarum* NRRL B-4496 – appartenenti alla Collezione DEBB, sono stati analizzati per la loro abilità nel produrre acido lattico, allo scopo di identificare il ceppo con le migliori prestazioni da applicare nei processi di conversione dell'idrolizzato BSG in acido lattico. L'analisi è stata condotta nel mezzo sintetico MRS (Man, Rogosa, and Sharpe, Oxoid), noto per essere un terreno ricco in grado di soddisfare le esigenze nutritive dei batteri lattici, incapaci di sintetizzare vitamina B ed amminoacidi. Durante le 96 ore di fermentazione sono stati monitorati la crescita cellulare, espressa come concentrazione di biomassa secca sul volume totale (g/L), il consumo di glucosio e la produzione di acido lattico.
I risultati hanno mostrato notevoli differenze nella crescita cellulare dei ceppi analizzati, variando da un valore minimo di 1.9 ± 0.5 g/L per *L. acidophilus* ATCC 4356 ad un valore massimo di 4.56 ± 0.5 g/L mostrato da *L. lactis* INRA 18. Per quel

che concerne la produttività volumetrica, è stato osservato un minimo di 0.22 g/L h per *L. pentosus* NRRL B-227 ed un massimo di 0.50 g/L h per *L. acidophilus* ATCC 53672. L'analisi dell'$Y_{P/S}$, indice della quantità la quantità di acido lattico prodotto per glucosio consumato, rapportato alla massima resa teorica di acido lattico (1 g/g), ha mostrato un valore minimo di 0.52 g/g per il ceppo *L. pentosus* NRRL B-227 ed un massimo di 0.99 g/g per *L. acidophilus* ATCC 43121, seguito dai ceppi *L. acidophilus* ATCC 53672, *L. plantarum* NRRL B-4496, *L. acidophilus* ATCC 4356, e *L. lactis* INRA 18 con un valore di $Y_{P/S}$ di 0.98 g/g, 0.96 g/g, 0.95 g/g, e 0.70 g/g, rispettivamente.
Tra i ceppi analizzati, *L. acidophilus* ATCC 43121 è stato selezionato per la conversione dall'idrolizzato AAT BSG1 ed AAS BSG2, avendo mostrato la massima produzione di acido lattico, pari a 16.1 g/L, in 48 ore di fermentazione con una produttività volumetrica di 0.34 g/L h.

- **Identificazione e parziale caratterizzazione di un cocktail (emi)cellulolitico dal fungo *Pleurotus ostreatus***

Al fine di identificare una fonte di carbonio in grado di indurre la produzione di cellulasi e xilanasi nel ceppo fungino *Pleurotus ostreatus*, sono state allestite crescite in presenza di differenti fonti di carbonio, quali carbossimetilcellulosa, cellulosa microcristallina, cellobiosio, soforosio, xilano, xilosio, xilitolo, arabinoxilano, galattosio e lattosio. L'andamento nel tempo relativo alla produzione di attività cellulasica e xilanasica ha evidenziato l'effetto induttivo della cellulosa microcristallina, grazie alla quale è stato ottenuto un picco massimo di produzione di 3.19 U/mL e 51.32 U/mL per cellulasi e xilanasi, rispettivamente, al 9° giorno di fermentazione.
Espermenti di zimografia accoppiati ad analisi di proteomica hanno permesso di identificare 7 peptidi in corrispondenza dell'alone di attività xilanasica aventi omologia con la xilanasi HB188 da *P. ostreatus*, (identification number NCBI: gi 164562254). Riguardo l'attività cellulasica, in corrispondenza del primo alone di attività sono stati identificati 17 peptidi aventi omologia con l'1,4-β-cellobiosidasi (cbhI-1) da *Pleurotus Florida* (gi 146350520), mentre in corrispondenza del secondo alone di attività sono stati identificati 8 peptidi aventi omologia con l'1,4-β-cellobiosidasi (cbhI-3) da *P. Florida* (identification number NCBI: gi 146424871).
Al fine di determinare le migliori condizioni di reazione da testare durante la saccarificazione della biomassa lignocellulosica di *Arundo donax*, è stata condotta una parziale caratterizzazione della mix enzimatica, determinando la temperatura e pH ottimali per l'attivita catalitica, la termoresistenza e la stabilità a pH. Gli enzimi sono risultati essere stabili in un ampio range di pH, mostrando massima attività cellulasica e xilanasica a pH 4.0 e 5.3, rispettivamente; entrambi gli enzimi presentano un optimum di attività alla temperatura a 50° C, temperatura a cui solitamente vengono condotte le reazioni di saccarificazione. Lo studio della termostabilità ha mostrato come l'emivita della cellulasi prodotta da *P.ostreatus* è di 7 ore sia a 30 °C che a 40 °C, mentre quella della xilanasi è di 2 giorni a 30 °C e 7 ore a 40 °C.

Sviluppo di processi di conversione delle biomasse lignocellulosiche in prodotti ad alto valore aggiunto

- **Ottimizzazione statistica del processo di idrolisi della biomassa di *Arundo donax* mediante il cocktail enzimatico da *Pleurotus ostreatus***

La mix enzimatica, costituita da cellulasi e xilanasi, prodotta dal fungo *Pleurotus ostreatus* è stata utilizzata in esperimenti di ottimizzazione statistica del processo di saccarificazione della biomassa lignocellulosica di *Arundo donax*. L'obiettivo è stato quello di ottenere la massima resa di zuccheri fermentabili, mediante l'identificazione delle variabili maggiormente influenti sul processo e la determinazione della loro combinazione ottimale. Durante gli esperimenti di ottimizzazione, la mix fungina è stata utilizzata in combinazione ad una mix di enzimi commerciali ad attività cellobiasica e β-xilosidasica (145U gds^{-1} di cellobiasi da *Aspergillus niger* e 8 U gds^{-1} di β-xilosidasi termostabile), prodotte a bassi livelli dal fungo, ma necessarie alla completa idrolisi della componente polisaccaridica. In primo luogo, mediante l'analisi Plackett-Burman, sono stati identificati i paramenti operativi che maggiormente influenzano la resa di zuccheri idrolizzati ottenibili a fine processo. Lo studio è stato condotto su 11 variabili, quali temperatura, concentrazione della biomassa, pH, tempo di incubazione, unità di cellulasi gds^{-1} da *P. ostreatus*, unità di cellulasi gds^{-1} da *Trichoderma reesei* ATCC26921 e 5 variabili fittizie; queste ultime sono state inserite nell'analisi come controllo interno al fine di ottenere una validazione dei risultati. Per ciascuna variabile sono stati considerati 2 differenti valori, uno massimo ed uno minimo combinati in 15 differenti esperimenti. Ciò ha consentito di identificare, nell'ordine, temperatura, pH e tempo quali parametri maggiormente influenti sulla resa del processo di idrolisi della biomassa di *A. donax*. Successivamente, l'analisi fattoriale 3^3 ha permesso di definire la migliore combinazione di tali parametri, al fine di massimizzare ulteriormente la resa in zuccheri fermentabili. Considerando 3 valori (uno massimo, uno minimo ed uno intermedio per le variabili temperatura, tempo e pH, ed il valore medio rispetto a quelli dell'analisi Plackett-Burman per le variabili risultate trascurabili, è stato ottenuto un pannello di esperimenti costituito da 30 differenti combinazioni. Il massimo risultato di 480.10 mg di zuccheri gds^{-1} è stato ottenuto ad una temperatura di 45 °C e pH 3.5 dopo 96 ore di incubazione. Inoltre dall'analisi è emerso comel'interazione tra le variabili temperatura e tempo eserciti un forte effetto positivo sulla resa del processo. E' stato osservato, infatti, che mantenendo costante il pH al valore di 3.5, la resa in zuccheri incrementa nell'intervallo di temperatura tra i 35 °C ed i 45 °C, andamento maggiormente evidente in corrispondenza delle 96 ore di incubazione. Dopo tale tempo, ulteriori incrementi di temperatura determinano un riduzione della resa di saccarificazione.

I risultati ottenuti hanno mostrato come la mix enzimatica ad attività (emi)cellulolitica prodotta dal fungo *P. ostreatus* possa essere utilizzata nel processo di idrolisi della biomassa *A. donax*, arrivando ad ottenere una resa di zuccheri comparabile, in alcuni casi, a quelle ottenute utilizzando mix enzimatiche commerciali.

- **Produzione di etanolo di seconda generazione dagli scarti della produzione della birra**

L'idrolizzato AAT BSG1, ottenuto mediante pretrattamento acido-alcalino e saccarificazione dello scarto proveniente dal birrificio Bier Hoff (Curitiba-PR, Brasile), è stato utilizzato come substrato per la fermentazione del ceppo *S. cerevisiae* NRRL YB 229, precedentemente selezionato come il miglior produttore di etanolo in mezzo

sintetico. E' stata, quindi, valutatata sia la capacità di crescita del ceppo che l'abilità a produrre etanolo utilizzando un idrolizzato prodotto a partire da uno scarto lignocellulosico. L'idrolizzato AAT BSG1 è stato diluito per ottenere una concentrazione finale di 50 g/L di glucosio ad un pH di 6.0. I risultati hanno dimostrato le potenzialità dell'idrolizzato AAT BSG1 come mezzo di fermentazione per la produzione di etanolo, sia in assenza che in presenza di estratto di lievito, utilizzato come fonte di azoto. Sebbene in entrambi i terreni di fermentazione sia stata raggiunta una concentrazione simile di etanolo, è importante osservare che l'aggiunta dell'estratto di lievito ha determinato un aumento della produttività, favorendo l'assorbimento di glucosio e la crescita cellulare. Infatti, nell'idrolizzato AAT BSG1 arricchito con estratto di lievito è stata osservata una produttività di 0.53 g/L·h, valore quasi due volte maggiore agli 0.25 g/L·h ottenuti in assenza della fonte di azoto. Nell'idrolizzato arricchito con estratto di lievito, la massima produzione di etanolo, pari a 12.79 ± 1.2 g/L e corrispondenti a 0.28 g di etanolo per g di glucosio (55% della massima resa teorica), è stata ottenuta dopo 24 ore, mentre in assenza di una fonte aggiuntiva di azoto una produzione simile, pari a 12.0 ± 1.2 g/L corrispondenti a 0.26 g di etanolo per g di glucosio (55 % della massima resa teorica), è stata ottenuta soltanto dopo 48 ore di fermentazione.

- **Produzione di acido lattico dagli scarti della produzione della birra**

Gli idrolizzati AAT BSG1 ed AAS BSG2, ottenuti dal pretrattamento e saccarificazione del BSG1 (birrificio Bier Hoff, Curitiba, Brasile) e del BSG2 (birrificio Maneba Striano, Italia), sono stati utilizzati come substrato per la produzione di acido lattico ad opera del batterio *Lactobacillus acidophilus* ATCC 43121, precedentemente selezionato come miglior produttore di acido lattico in mezzo sintetico. Gli idrolizzati BSGs, sia tal quali che addizionati con estratto di lievito, sono stati diluiti per ottenere una concentrazione finale di glucosio di 50 g/L ad un pH di 6.0. I risultati hanno mostrato che, in presenza della fonte di azoto, la resa di acido lattico da idrolizato AAT BSG1 è stata incrementata da 0.48 g/g ad un valore di 0.60 g/g (con un'efficienza di conversione del glucosio in acido lattico pari al 65 %) e da 0.52 g/g ad un valore di 0.60 g/g utilizzando l'idrolizzato AAS BSG2. Anche la produttività volumetrica è risultata essere maggiore in presenza di estratto di lievito; infatti, in 48 ore di fermentazione, l'utilizzo dell'idrolizzato AAS BSG2 addizionato con estratto di lievito ha permesso di ottenere la concentrazione massima di acido lattico di 22.16 g/L, valore 27 % più elevato di quello ottenuto in assenza della fonte di azoto. Sebbene la migliore produzione di acido lattico sia stata ottenuta utilizzando l'idrolizzato AAS BSG2, anche nel caso dell'idrolizzato AAT BSG1 l'aggiunta della fonte di azoto ha consentito di ottenere una concentrazione di acido lattico di 12.26 g/L, valore superiore agli 8.9 g/L osservati in assenza dell'estratto di lievito. E' possibile che l'addizionata fonte di azoto abbia permesso di raggiungere un rapporto carbonio/azoto ottimale sia per la crescita del ceppo *L. acidophilus* ATCC 43121 che per la produzione di acido lattico.

Conclusioni

In sintesi questo progetto di dottorato ha previsto:

- la selezione di nuovi biosistemi appartenenti alla collezione DEBB, quali il ceppo *Aspergillus niger* NRRL 3312 ad attività (emi)cellulolitica ed il ceppo *Pleurotus sajor-caju* INRA 3501 ad attività ligninolitica, entrambi applicati in processi di fermentazione su stato solido su BSG per la produzione a basso costo di enzimi di interesse; inoltre, sono stati selezionati il batterio lattico *Lactobacillus acidophilus* ATCC 43121 ed il ceppo etanologenico *Saccharomyces cerevisiae* NRRL YB 2293, testati nei processi di conversione dell'idrolizzato BSG in acido lattico ed etanolo, rispettivamente;

- lo sviluppo di nuovi biocatalizzatori, un cocktail (emi)cellulolitico da *Pleurotus ostreatus* su cui è stata condotta una parziale caratterizzazione, ed un cocktail (emi)cellulolitico da *Aspergillus niger* NRRL 3312, le cui abilità idrolitiche saranno testate allestendo processi di saccarificazione per la conversione della lignocellulosa in zuccheri fermentabili;

- l'ottimizzazione statistica del processo di saccarificazione della biomassa lignocellulosica *Arundo donax* mediante l'utilizzo del cocktail (emi)cellulolitico da *Pleurotus ostreatus*;

- lo sviluppo di un processo di conversione dell'idrolizzato, ottenuto dal pretrattamento e saccarificazione dello scarto lignocellulosico BSG, in acido lattico mediante l'applicazione del batterio *Lactobacillus acidophilus* ATCC 43121;

- lo sviluppo di un processo di conversione dell'idrolizzato, ottenuto dal pretrattamento e saccarificazione dello scarto lignocellulosico BSG, in etanolo mediante l'applicazione del ceppo *Saccharomyces cerevisiae* NRRL YB 2293.

List of Abbreviations

ABTS: 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid)
AFEX: Ammonia Fiber Explosion
AXE: Acetyl Xylan Esterase
AZO-CMC: Azo-CarbossiMetilCellulosa/Azo-CarboxyMethylCellulose
BG: β-glucosidases
BSG: Brewers' Spent Grain
CAZY: Carbohydrate-Active EnZYmes
CBH: cellobiohydrolases
CBP: Consolidated BioProcessing
CE: Carbohydrate Esterases
CMC: CarbossiMetilCellulosa/CarboxyMethylCellulose
DNS: dinitrosalicylic acid reagent
EG: Endoglucanases
EI: Enzymatic Index
EXs: endo-β-xylanases
FAE: Feruloyl Esterase
gds: grams of dry substrate
GH: Glycoside Hydrolases
GHGs: Greenhouse Gas Emissions
GRAS: Generally Recognized As Safe
HMF: hydroxymethylfurfural
IEA: International Energy Agency
LDA: Lignin Degrading Auxiliary enzymes
LiP: Lignin Peroxidase
LO: Lignin Oxidases
MnP: Manganese Peroxidase
PL: Polysaccharide Lyases
PVC: Polyvinyl Chloride
SDS-PAGE: Sodium Dodecyl Sulphate-PolyAcrylamide Gel Electrophoresis
SHF: Separate Hydrolysis and Fermentation
Smf: Submerged fermentation
SSCF: Simultaneous Saccharification And Co-Fermentation
ssf: simultaneous saccharification and fermentation
SSF: Solid State Fermentation
US FDA: Food and Drug Administration
VP: Versatile Peroxidase

Chapter I

Introduction

1.1 Lignocellulosic biomasses as a renewable alternative to fossil-based resources

Currently, the worldwide energy generated derives almost exclusively from petrochemical feedstock. It was estimated by the International Energy Agency (IEA) that, in 2014, primary sources of energy consisted of petroleum (39.9 %), coal (11.5 %), natural gas (15.1 %) and others (33.5 %) [1]. The main problem related to the fossil-based resources is their limitation in supply due to the non-renewability; indeed, it was calculated that depletion times for oil, coal and gas are of about 35, 107 and 37 years, respectively. This means that coal reserves will be available up to 2112, and will be the only fossil fuel source remaining after 2042 [2]. The shortage of energy fossil sources, the global warming, the high release of greenhouse gas emissions (GHGs) and the waste recycling problem prompted an increasing worldwide interest in alternative renewable sources of energy. The European Parliament with the DIRECTIVE 2009/28/CE - also known as "Climate-Energy package 20-20-20" - establishes national renewable energy targets that result in an overall binding target of a 20 % share of renewable energy sources in energy consumption by 2020 and a binding 10 % minimum target for renewable fuels in transport to be achieved by each Member State.

Lignocellulosic biomasses derived from dedicated crops and agro-industrial residual materials are the most promising renewable sources to be used as alternatives to the fossil-based ones, minimizing the problematics of the modern industrial societies [3]. They are inexpensive and largely available around the world. The large-scale cultivation of dedicated crops, like the perennial biomass *Arundo donax*, in marginal lands non appropriate for the traditional food crops is spreading throughout the world, limiting the competition between the food- and the non food-crop lands, and improving the soil fertility [4]. On the other hand, lignocellulosic agro-industrial residual materials represent an alternative cheap source that allow to further minimize the conflict food *versus* fuel, avoiding the displacement of food crops and the issues related to the deforestation [5]. The lignocellulosic biomasses consist of mainly three polymers - cellulose, hemicellulose and lignin - organized into complex amorphous three-dimensional structures, with a different relative composition depending on the specie (Fig. 1). The cellulose, composed of (1,4)-D-glucopyranose units linked through β-1,4 glycosidic bonds, is the major component of lignocellulosic biomass, accounting for about 40-50 % of the weight of dry wood. Hemicellulose, a mixture of various monosaccharides, such as xylose, arabinose, mannose and galactose, organized in a heterogeneous and ramified polymer, is the second most abundant polymer of lignocellulosic biomass, accounting for 25-35 % of total dry wood. At last, lignin represents the smallest heterogeneous polymer of the lignocellulosic biomass, with a 10-25 % of total dry wood and it consists of phenyl-propane, methoxy groups and noncarbohydrate poly phenolic substance, commonly linked by ether bonds [6]. Table 1 shows the composition of some common lignocellulosic feedstocks. Due to the high cellulose and hemicellulose contents, the lignocellulosic biomasses can be converted into a wide range of added value bioproducts, such as enzymes, biofuels, organic acids, biopolymers, bioelectricity, and molecules for food and pharmaceutical industries. Many biotechnological processes are being developed and optimized in order to obtain eco-friendly and cost-effective bioproducts that meet the world's energy and chemicals needs, as describes in the paper "**Waste valorization by biotechnological conversion into added value products**" (See Appendix IV - Other publications).

Table 1: Percentages of cellulose, hemicellulose and lignin dry weight content in common lignocellulosic biomasses.

Lignocellulosic biomass	Lignin (%)	Hemicellulose (%)	Cellulose (%)
Sugar cane bagasse	20	25	42
Corn stover	19	26	38
Brewers' spent grain	12	34	14
Eucalyptus	24	25	45
Newspaper	18-30	25-40	40-55
Wheat straw	16-21	26-32	29-35
Corn cobs	15	35	45
Rice straw	18	24	32
Sweet sorghum	21	27	45
Bagasse	23	16	54

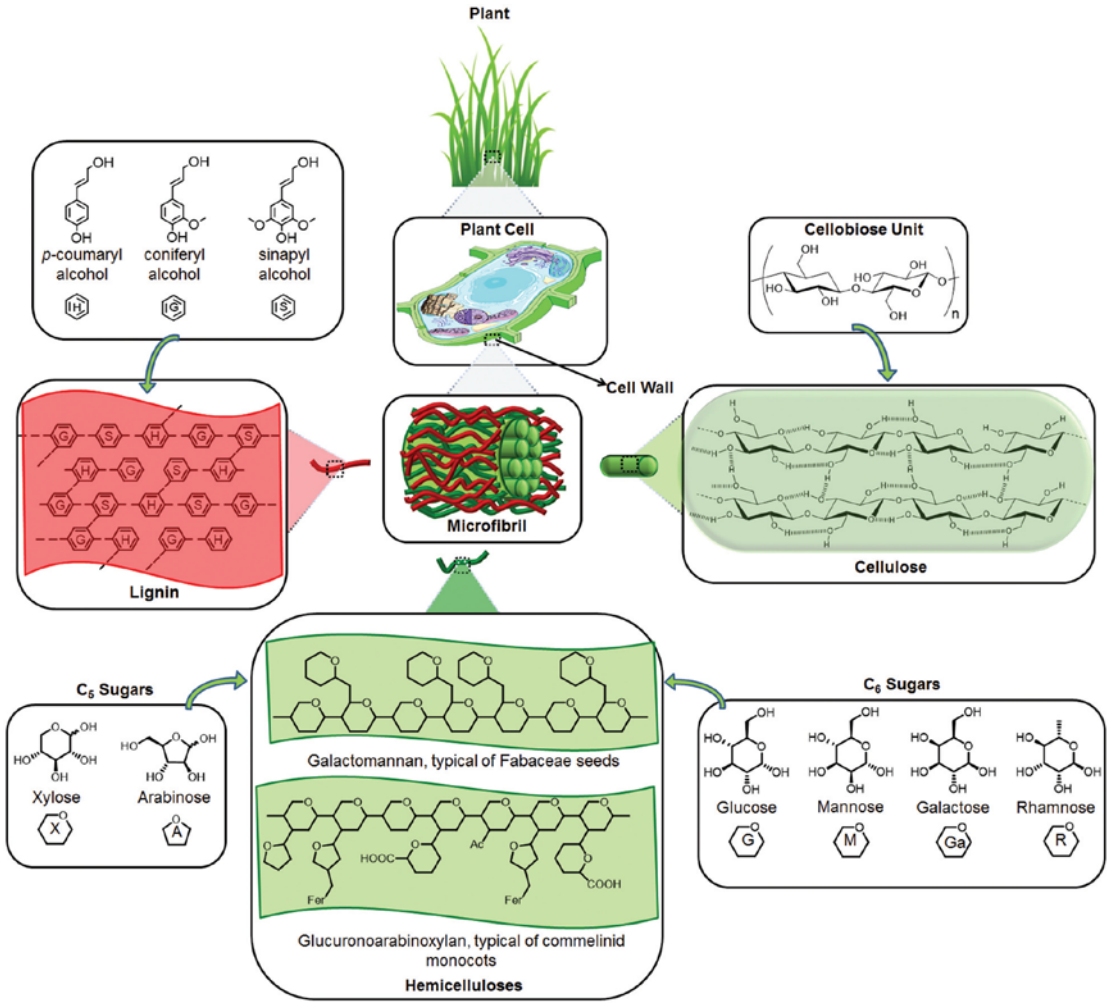


Figure 1. Macromolecular structure of lignocellulosic biomass. Cellulose microfibrils are embedded into a matrix consisting of lignin and hemicellulose in the cell wall.

1.2 Conversion process for lignocellulosic wastes upgrading into added value products

Due to the complexity of the lignocellulose structure, the conversion process into added value bioproducts commonly requires the following main steps: **i)** a pretreatment to remove the recalcitrant lignin; **ii)** the enzymatic hydrolysis of the cell wallpolysaccharides into monomeric sugars by using enzymatic cocktails mainly composed of cellulases and hemicellulases, and **iii)** the fermentation of both exoses and pentoses to generate upgraded bioproducts (Fig. 2). These three steps are largely described in the paper "**The second generation ethanol production**" (See Appendix IV - Other publications), with a particular focus on the main routes to produce bioethanol from lignocellulosic crops.

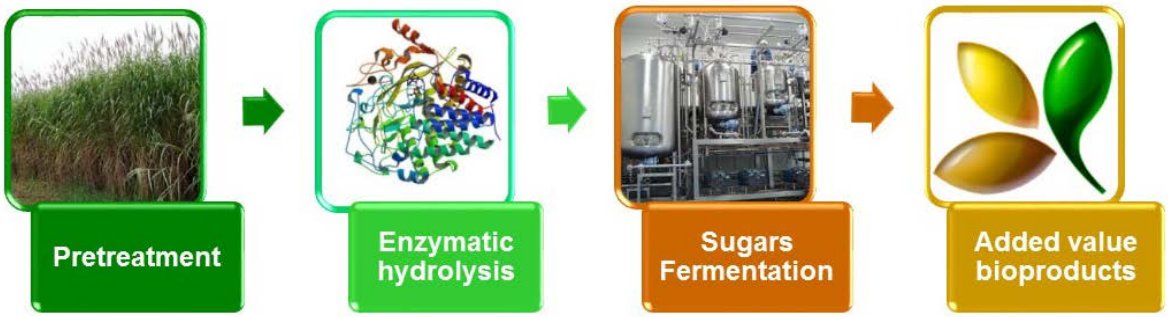


Figure 2. Main steps of lignocellulosic biomass upgrading into added value bioproducts.

1.2.1 Pretreatment

An effective pretreatment of lignocellulosic biomasses is aimed at increasing the accessibility and digestibility of the (hemi)cellulose fraction, thus improving the subsequent enzymatic hydrolysis. Extensive researches are focusing on pretreatment optimization, in order to develop processes that maximize lignin removal and minimize sugars loss, toxic compounds generation and overall operating costs.

The pretreatment of lignocellulosic biomasses are mainly classified into physical, chemical and biological methods. The physical pretreatment increases the surface area by reducing the size of the biomass; the chemical methods remove and/or dislocate the hemicellulose and lignin, thus, loosening the structural network of lignin and holocellulose, whilst the biological pretreatment mainly causes the delignification of lignocellulosic biomass.

1.2.1.1 Physical, chemical and physico-chemical pretreatment

Physical treatments enhance the digestibility of (hemi)celllulose component through the reduction of particles size of lignocellulosic material; the required energy depends on the final particle size and the macromolecular structure of the feedstock [33]. Among all the physical treatments, pyrolysis, microwave and extrusion are known as the most efficient and cost-effective ones. The pyrolysis involves the decomposition of cellulose into H_2 and CO by using temperatures higher than 300 °; the microwave treatment leads to the degradation of lignin through the transfer of heat from waves to organic molecules [9]. The extrusion, that allows to mix, heat and shear the lignocellulosic biomasses, is receiving great attention since the process does not

generate furfural, hydroxymethylfurfural (HMF) and effluent and it can be carried out in a continuous operation mode [34]. Chemical processes are the major studied methods for the lignocellulose pretreatment and they involve the use of a wide range of chemical agents [13]. Among them, acid and alkaline treatment, organosolv and ozonolysis are the most well-known. The acid treatment promotes the hemicellulose hydrolysis, involving the use of concentrated or diluted acids (usually between 0.2 % and 2.5 % w/w) at temperatures between 130-210 °C [36]. In the alkaline pretreatment, biomass is treated with alkali such as sodium, potassium, calcium and ammonium hydroxides at normal temperature and pressure; it is considered a slow process in comparison with the other ones, requiring hours or days rather than minutes or seconds [37]. The organosolv treatment is based on the use of organic solvent - ethanol, methanol, acetone - or mixtures of them in combination with water, that causes the breach of the internal bonds in lignin and also of those between lignin and hemicellulose [38]. The ozonolysis treatment attacks the aromatic rings of the lignin structure without damaging hemicellulose and cellulose [9]. In order to increase the efficiency of the process, a combination of physico-chemical treatments have been recently considered (Fig. 3); among the several developed methods, the steam explosion and the ammonia fiber explosion (AFEX) are the major investigated [39]. During the steam explosion, the biomass is subjected to a combination of high temperatures (180 to 240 °C) and pressures (from 145 to 435 psi) for few seconds to several minutes, followed by a quickly pressure reduction, causing the hemicellulose degradation and lignin transformation [40].

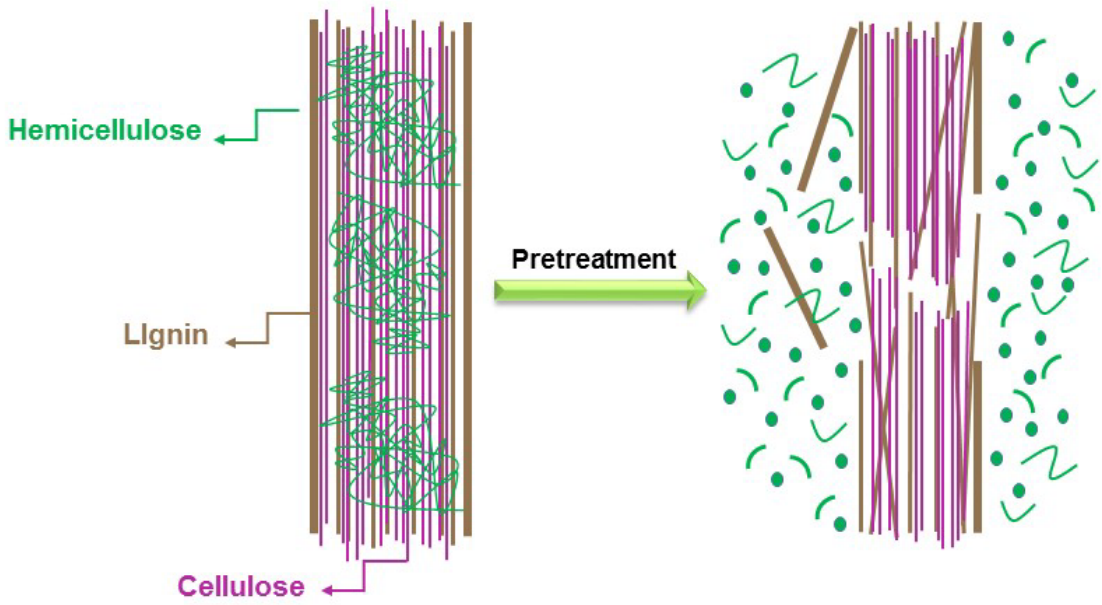


Figure 3. Schematic effects of the physico-chemical pretreatment on the lignocellulosic structure.

The AFEX treatment involves the use of ammonia at high temperatures (60-100 °C) and pressures (250-300 psi) for 5 min, followed by a rapid decrease of pressure; it causes the modification of lignocellulosic structure with a de-crystallization of cellulose, whereas the macromolecular composition remains unchanged [41].
Although physico-chemical processes are the most largely applied lignocellulosic pretreatment methods, they cause environmental damages due to the high energy and expensive chemical compounds required. Moreover, toxic substances that

interfere with both (hemi)cellulolytic enzymatic activities and microbial fermentation, are generated during the process [7]. This requires an additional detoxification step that further increases the wastewater production and the overall costs of the process.

1.2.1.2 Biological pretreatment

The biological treatment is considered a promising approach for delignification of lignocellulosic biomass, since it does not require additional energy or chemicals, minimizing the negative impact on the environment [8]. Nevertheless, the process has not been largely applied because it requires a long time (about 3–5 months), results in very high feedstock loss and the rate of following hydrolysis is very low [42]. In the last decade, many efforts were focused on the biological treatment operative parameters optimization in order to maximize the delignification rate and, at the same time, the yield of the obtainable added-value bioproducts [43,44,45]. Biological pretreatment methods can be divided into three categories: fungal, microbial and enzymatic delignification.

The best effective fungal strains that can be employed in the biological delignification are brown and white rot fungi, which synthesize high level of ligninolytic enzymes. Brown rot fungi attacks cellulose while white and soft rots fungi attack both cellulose and lignin [9].

Figure 4. *Pleurotus ostreatus* on Brewers' spent grain

The Solid State Fermentation (SSF) through white-rot Basidiomycetes is the most investigated method for the biological biomass delignification (Fig. 4). Besides its numerous advantages, such as the use of low temperatures and pressures and no water and chemical agents requirement [46], the SSF also prevents the formation of toxic compounds that could inhibit the following hydrolysis and fermentation steps.

A single bacterium or a bacterial consortium have been widely investigated for their ability to delignify lignocellulosic biomasses [8,47]. In comparison to the fungi, the bacterial strains show lower level of (hemi)cellulolytic activity production, reducing the loss of cellulose with a significant advantages in the recovery of fermentable sugars [48,49]. Cultures composed of different known or unknown bacterial strains, thanks to their great self-evolution abilities and adaption, allow to perform the treatment with less restriction of the operative conditions such as pH, sterilization, temperature and nutrients [26].

The biomass delignification carried out by ligninolytic enzymes - laccases, lignin peroxidases (LiP), manganese peroxidases (MnP) and versatile peroxidases (VP) - is considered a feasible biomass treatment in alternative to the use of fungal and microbial strains [50,51]. Higher delignification rate and quicker process are two of the main advantages that the use of enzymes gives in comparison to the microorganisms [11]. Moreover, the enzymatic delignification could be carried out in a wide temperature and pH ranges, the nutrient supplementation is not required and there is not sugars consumption [52]. The main bottelneck is represented by the high cost of the enzymes and, for this reason, many researches are aimed at producing customized enzyme systems with improved hydrolysis efficiencies. Recently, the

interest is moving towards the expression of optimized enzymes in a cell factory; indeed, the use of one microorganism engineered with genes from various sources allows to reduce the impact from an economic point of view [53].

1.2.2 Hydrolysis

After the biomass pretreatment, saccharification of the accessible (hemi)cellulose component is carried out. Among the several developed methods (thermal, chemical and biochemical), the enzymatic hydrolysis is generally preferred due to the mild operation conditions, higher conversion yields, low energy cost, and less corrosive and toxic conditions in comparison to to the other hydrolysis methods [54].

Due to the complexity of the lignocellulosic structure the activities of multiple hydrolytic enzymes are required for a complete deconstruction of the cellulose and hemicellulose components. Hydrolytic enzymes are produced as either free cellulases or complexed cellulases, called cellulosomes [55]. Cellulosome systems are multi-enzymatic complexes, consisting of two subunit types, the scaffolding and the enzymatic subunits. The structural scaffolding subunits contain a carbohydrate-binding module that binds the cellulose surface and multiple copies of cohesins (named dockerins) that interact with the enzymatic subunits, Cellulose Binding Domains (CBD) and Carbohydrates Binding Modules (CBM).

According to Carbohydrate-Active EnZYmes (CAZY) [56] and Fungal Oxidative Lignin Enzymes (FOLy) databases [57], lignocellulose-degrading enzymes belong to Glycoside Hydrolases (GH), Polysaccharide Lyases (PL), Carbohydrate Esterases (CE), Lignin Oxidases (LO), and Lignin Degrading Auxiliary enzymes (LDA) families.

Among the GH, a wide spread group of enzymes which hydrolyse the glycosidic bond between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety carbohydrate moiety, three major classes can be found: the GH, a wide spread, are divided in three major classes: endoglucanases, exoglucanases and β-glucosidases [58]. Endoglucanases (EG) or 1,4-β-glucanases (EC 3.2.1.4) randomly hydrolyze intramolecular β-1,4-glucosidic linkages, creating new chain-ends; exoglucanases or cellobiohydrolases (CBH) (EC 3.2.91) hydrolyze the 1,4-glycosidic linkages from the reducing or non–reducing ends to form cellobiose; β-glucosidases (BG) (EC 3.2.1.21) hydrolyze cello-oligosaccharides and cellobiose into glucose eliminating cellobiose inhibition.

In addition to the three major groups of cellulose enzymes, there is also a number of hemicellulases which hydrolyze the hemicellulose component. Due to the more varied composition of this polysaccharide, enzymes degrading hemicellulose are divided into two major groups: depolymerising enzymes, which cleave the backbone, and enzymes that remove substituents. Among all the hemicellulases, most studies were focused on enzymes that hydrolyze xylan, such as endo-β-xylanases (EXs) and β-xylosidases. Endo-1,4-β-xylanase (1,4-β-D-xylan xylanohydrolases, EC 3.2.1.8) cleaves the glycosidic bonds in the xylan backbone releasing shorter xylo-oligosaccharides; β-xylosidase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.37) cleaves the small xylo-oligosaccharides into xylose.

The (hemi)cellulolytic enzymes are produced by several microorganisms such as fungi, bacteria, yeast, and marine algae. Filamentous fungi are interesting producers because the enzymes are secreted at levels higher than those of yeasts and bacteria [59,60].

Moreover, in order to completely convert the polysaccharides into fermentable sugars (xylose, mannose, arabinose), also several accessory enzymes are needed, such as acetyl xylan esterases (AXE, EC 3.1.1.72), feruloyl esterases (FAE, EC 3.1.1.73),

glucuronoyl esterases, α-L-arabinofuranosidases (EC 3.2.1.55), α-glucuronidases and mannanases [61].

1.2.3 Fermentation

Once lignin has been removed and the saccharification of the (hemi)cellulose portions has been carried out, the final step to be performed is the fermentation of sugars into the desired added value products. Two main routes can be followed for added value products generation, such as Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (ssf).
In SHF, the bioconversion of lignocellulose takes place in two separate reactors, thus separating the saccharification and the fermentation processes, resulting in the quick and easy possibility to optimize each production step, with conduction of each process at the optimal conditions of pH and temperature [12].
In ssf, enzymatic hydrolysis and fermentation are carried out together, using a unique reactor. By taking place in the same reactor, this kind of process has a number of advantages, such as the reduction of costs and the increase of hydrolysis rate, besides the possibility to make the fermentable sugars immediately available to micoorganisms. However, the ideal pH or temperature conditions for the saccharification step may differ from those of the fermentation; thus, it is very difficult to find favorable conditions for both processes [62].
On the other side, Consolidated BioProcessing (CBP) can provide an important contribution to reduce the costs of the bioconversion process, due to the use of a single engineered microorganism, able to directly convert cellulose into a value added product of interest by using its own enzymatic machinery.

1.2.3.1 Ethanol fermentation

A biological conversion of hexoses and pentoses, derived from a saccharification of pretreated lignocellulosic biomasses, into ethanol could be carried out by a variety of microorganisms, such as bacteria, yeast, or fungi. *Saccharomyces cerevisiae* is the most widely used organism for ethanol production from hexoses [62], whilst *Pichia stipitis* and *Candida shehatae* are the main yeasts able to ferment both hexose and pentose sugars to ethanol [63]. Bacteria belonging to the species *Clostridia* and *Zymomonas* [64], and fungi such as *Fusarium* spp. have been also demonstrated to be useful for ethanol production [65].
Since during the pretreatment and hydrolysis of lignocellulosic biomasses, many toxic compounds, that could inhibit the fermentation step, are generated [42], development of inhibitor-tolerant strains of microorganisms could be one of the approaches to improve the second generation ethanol production [66].
Besides the SHF and ssf, CBP has been so far recognized as the best system to reduce the cost of biomass processing, by combining the hydrolysis of the polysaccharides and the subsequent fermentation of the hexose/pentose sugars (Fig. 5). There are two main routes to perform CBP: category I is based on the use of a cellulase producing microorganisms, mainly fungi like *Trichoderma, Aspergillus*, *Rhizopus* and *Fusarium* engineered to ferment sugars into added value products with high titers of conversion [67], while category II uses ethanologenic microorganisms, both yeasts (*Trichoderma, Aspergillus*, *Rhizopus* and *Fusarium*) and bacteria (*Escherichia coli* and *Zymomonas mobilis*) engineered to be cellulolytic [68].

1.2.3.2 Lactic acid fermentation

The sustainable production of lactic acid through fermentation of lignocellulosic hydrolysates is predicted to significantly replace the chemical synthesis route based on the use of fossil resources [69]. The overall goal of the lactic acid fermentation process is to increase the product yield and productivity, improving the end-product recovery.

Fungi (e.g. genus *Rhizopus*) and lactic acid bacteria are the most widely employed microorganisms for lactic acid production [18]. Although the lactic acid fermentation is relatively fast and easy, many efforts were focused on the selection of the most favorable operative conditions, such as temperature, pH, aeration and agitation, which vary depending on the microorganism used, in order to improve the lactic acid yield. Moreover, the choose of homofermentative or heterofermentative microorganims leads to the production of one of the two stereoisomers of lactic acid or to their racemic mixture, respectively [70].

Currently, the SHF is the main route utilized for the lactic acid production from lignocellulosic biomasses [25,23,71], even if many efforts are focusing on the development of new microbial strains suitable for lactic acid production throughssf [72,22,73].

Last advances in the lactic acid production from lignocellulosic hydrolysate via fermentation regard the development of a CBP through a thermophilic bacteria, named *Paenibacillus macerans* IIPSP3 (MTCC 5569). This strain is not only capable of hydrolysing cellulose to glucose but also further fermenting it to L-Lactic acid under aerobic conditions, without any growth inhibition in presence of lignin [74].

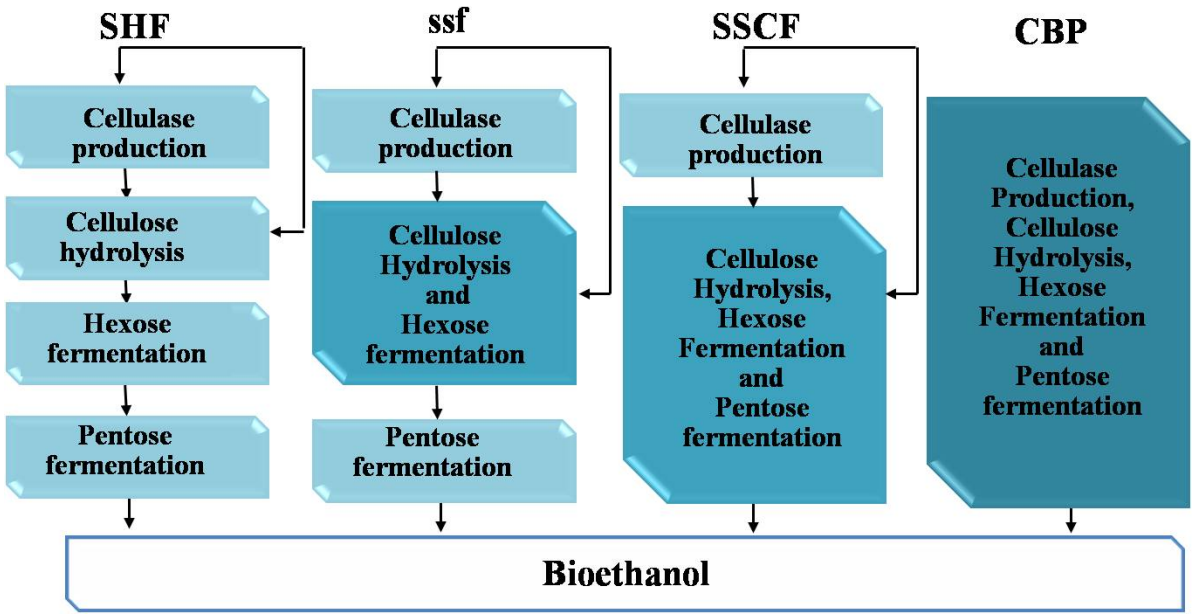


Figure 5. Processes of sugars and bioethanol production. SHF separate hydrolysis and fermentation, ssf simultaneous hydrolysis and fermentation, SSCF simultaneous saccharification and cofermentation of both hexoses and pentoses.

1.3 Strategies to improve lignocellulosic conversion into added value bioproducts

Although several advances have been achieved, the lignocellulosic biomass upgrading into added value products still shows issues that interfere with the development of a cost-effective and eco-friendly conversion process.

As far as concern the pretreatment, the need is to reduce and/or avoid the use of chemicals and high energy input, typically required by the most largely employed physico-chemical methods. Thus, the efforts were focused on the solid state fermentation as biological treatment, in order to reduce the process time together with the microbial and chemical contaminants, that can decrease the followed saccharification yield.
Moreover, it is worth noting that, the solid state fermentation by fungal strains have been also largely exploited as a process to produce (hemi)cellulolityc enzymes at low costs, due to the use of cheap raw materials as substrate [75,76]; indeed, many fungal strains are known to be good producers of lignin-degrading as well as (hemi)cellulolytic enzymes [77]. In table 2, a list of various lignocellulosic biomasses used as substrate for the production of different lignocellulolityc enzymes was reported.

Table 2. Various lignocellulosic biomasses used for the production of different microbial enzymes.

Lignocellulosic biomass	Microorganism	Enzymes produced
Corn cobs	*Trametes versicolor*; *Phanerochaete chrysosporium*, *Aspergillus niger*	MnP, LiP, laccase, protease, xylanase
Banana stalk	*Schizophyllum commune*, *Phanerochaete chrysosporium*, *Trametes versicolor*; *Pleurotus ostreatus*	MnP, LiP, laccase, xylanase, endoglucanase
Rice bran	*Aspergillus niger*	Protease
Wheat bran	*Aspergillus niger*, *Morchella sculenta*, *Fomes sclerodermeus*, *Trametes versicolor*	Protease, endoglucanase, β-glucosidase, laccase, MnP
Eucalyptus residue	*Lentinus edodes*	Xylanase, cellulase, MnP, laccase
Tomato Pomace	*Pleurotus ostreatus*, *Trametes versicolor*	laccase, protease

The low-cost enzymes production is the main challenge for the development of a competitive bioconversion process; indeed, actually, the hydrolysis step has the higher economic impact on the overall bioconversion process, truly due to the high lignocellulolytic enzymes costs [55].
For this reason, research is looking for new (hemi)cellulases with higher efficiencies, increased stability at elevated temperatures and at extreme pH values, and higher tolerance to end-products inhibition. A combination of genomic, transcriptomic and proteomic analysis allows to i) enhance enzymes from natural sources through rational design and directed evolution strategies [29], ii) discover new enzymes by metagenomic approach [30] and, iii) design artificial enzymes with suitable properties [78].
The enzymatic hydrolysis of lignocellulose is also affected by temperature, time, pH, enzyme loading, product concentration, and biomass structural features. Many studies were focused on the statistical optimization of pretreated biomass saccharification, in order to identify the most significant parameters and their combined effect on the sugars release [79,80,81].
Moreover, several efforts are under way to improve bioreactor systems adopted for the biomasses bioconversion process, in order to solve the environmental and

economical issues of the process [82,83]. The review "**Bioreactors for lignocellulose conversion into fermentable sugars for production of high added value products**" (See Appendix IV - Other publications), summarizes the last advances in the bioreactor field, with main focus on the new configurations and agitation systems, for conversion of dedicated energy crops and residual materials into sugars and ethanol by separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (ssf), simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP).

1.4 Integrated Biorefinery concept

Biorefinery, defined as "the sustainable processing of biomass into a spectrum of marketable products and energy" [84], should minimize the dependence on fossil-based resources and the generation of solid/liquid wastes, that was estimated, in the 2010, to be between 7 to 10 billion tonnes [85].

In the Biorefinery platform, where the upstream, midstream, and downstream processes for biomass conversion into fuels, power, and chemicals are integrated, the wastes is consided as a renewable resources that can be used again and again, replacing the exhaustible fossil-based sources. Indeed, in the biorefinery platform takes place the circular economy, based on the closed-loop system aimed at maximizing the recovery of raw materials derived from the waste at end-of-life [86]. In the manuscript **"Biological treatment processes for advancing waste biorefinery and advocating circular economy"** (See Appendix IV - Other publications), the last advances in the biological processes for lignocellulosic wastes upgrading were described, focusing the attention on the closed loop approach advocated by the circular economy and realized through the biorefinery platform. Moreover, biorefinery concept is based on the sustainability, taking into account the possible consequences due to the competition for food and biomass resources, the impact on water use and quality, changes in land-use and fertility, net balance of GHGs, impact on biodiversity, potential toxicological risks, and energy efficiency.

Depending on the type of raw materials used, the biorefinery were classified into whole crop biorefineries (first generation), lignocellulosic feedstock biorefineries (second generation), oleochemical biorefineries, green biorefineries, and marine biorefineries [87]. The first generation biorefinery uses sugar- or starch-rich edible crops, such as sugarcane and starch, determining the food versus fuel conflict, while the secon generation ones allow to avoid this conflict by using industrial and food wastes, and lignocellulosic feedstocks [88]. Oleochemical biorefineries uses fatty acids, fatty esters, and glycerol from oil crops to produce chemicals, lubricants, and surfactants [89]; green biorefineries produced water-soluble compounds (lactic acid, amino acids) and fibers starting from grass [90] and, the marine biorefineries use microalgae and macroalgae and their derived products as sources for the production of added value bioproducts [91].

After the first oil crisis, the first and the second generation biorefineries have undergone a considerably development, contributing to the increase of the renewable resources usage [92]. In 2013, the renewable energy/resource supplied a 19.1 % of the global energy request, and furthermore, this contribution is continued to expand in 2014 [93]. Biomass processing for energy purposes, such as ethanol and biodiesel generation, is concentrated in a small number of countries, including the USA, Brazil, Spain and the Netherlands. Currently the USA and Brazil are the major fuel ethanol producers, reaching the 87 % of the global fuel ethanol production [93]. In the USA, the biofuels industry has undergone significant expansion in recent years focusing

the efforts on the improvement of first-generation biofuels production; in Brazil, the great development of the sugar cane industry, that has led to the highest penetration worldwide of flex-fuel vehicles, now attracts additional investments in bio-based plastics, for example PVC (Polyvinyl chloride). In Europe the biorefinery platforma are not yet widely spread; thus, many efforts have been focused on the development of future biorefineries, through an improvement of a sustainable crops production, investment in agriculture, new public police incentives, training and cooperation initiatives [94].

1.5 Aims of the thesis

The overall aim of this PhD project is to improve technologies and biological tools for conversion of lignocellulosic biomass by eco-friendly and eco-efficient production of high added value bioproducts, such as lignocellulolytic enzymes, bioethanol and lactic acid. The exploited lignocellulosic biomasses involved both dedicated energy crops, cultivated on marginal land of the Campania region (e.g. *Arundo donax*), and lignocellulosic agro-industrial wastes abundantly discharged in EU (e.g. Brewers' spent grain). The activities of this PhD project were carried out in the frame of the project BIOASSORT, a Marie-Curie action aimed at the "Improvement of technologies and tools, e.g. biosystems and biocatalysts, for waste conversion to develop an assortment of high added value eco-friendly and costeffective bio-products"(318931).

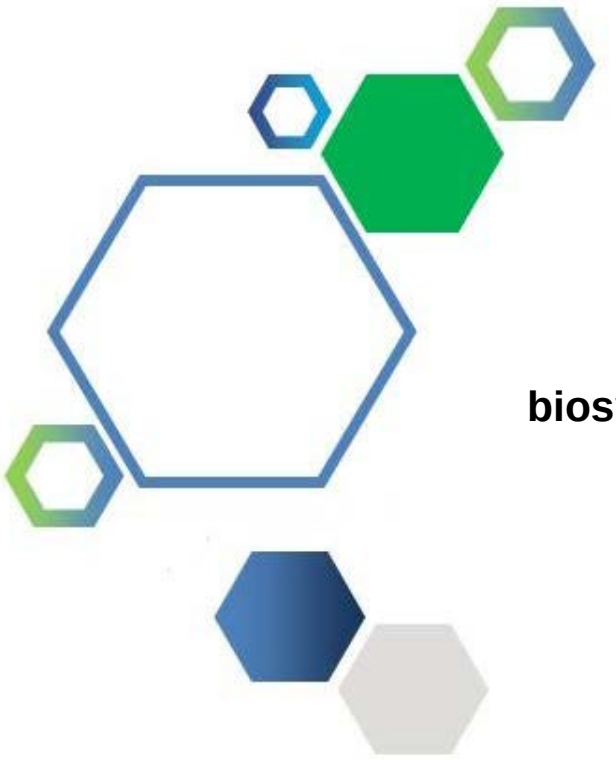
One of the main objective is aimed at enlarging the assortment of the patrimony of microbes for lignocellulosic biomass valorisation, achieved by:

- **i)** selection of lignocellulolytic, ethanologenic and lactic acid microorganisms belonging to the "Strain Collection of the Bioprocess and Biotechnology Division" from the Department of "Engenharia de Bioprocessos e Biotecnologia", University Federal do Paraná (DEBB), Curitiba-PR, Brasil (Bioassort's partner);
- **ii)** production of lignocellulolytic enzymes by the new selected microorganisms through solid state fermentation on lignocellulosic biomass;
- **iii)** identification and characterization of new enzymatic cocktails, containing (hemi)cellulolytic activities, produced by the new selected lignocellulolytic microorganisms;
- **iv)** exploitation of the fungal strain *Pleurotus ostreatus,* that was so far largely investigated in the laboratory where the activities of this PhD project were carried out, as a source of (hemi)cellulolytic enzymatic cocktails for (hemi)celluloses conversion.

On the other side, another main objective of this PhD project is aimed at setting up the conversion processes for the lignocellulosic biomass upgrading into ethanol and lactic acid, improving their production yields. The main outlines concerning this task are following reported:

- **i)** investigation of an efficient pretreatment for the agro-industrial residue Brewres' spent grain in order to remove lignin and improve accessibility of polisaccharides for the following enzymatic saccharification;
- **ii)** optimization of *Arundo donax* saccharification through the (hemi)cellulolytic enzymes produced by *Pleurotus ostreatus*;
- **iii)** development of fermentation processes by using the sugar mixture, obtained by enzymatic hydrolysis of pretreated Brewers' spent grain, as fermentation medium for the growth of the new ethanologenic and lactic acid microorganisms.

The operative goals previously described aim at the general objective of developing a new eco-friendly end cost-effective valorization process for the production of ethanol and/or lactic acid from lignocellulosic biomasses.



Chapter II

Development of new biosystems and biocatalysts for waste upgrading

2.1 Introduction

In nature, many microorganisms, including fungi and bacteria, are able to degrade cellulose and other plant cell wall fibres by using several lignocellulolytic enzymes. Biomass degradation is performed mainly by complex enzymatic mixtures consisting of cellulases [95], hemicellulases [96] and ligninases [97], acting in a synergistic manner due to the complexity of the lignocellulosic material.
Since 1976, over 14,000 fungal species capable of degrading cellulose have been isolated, but only few of them – such as ascomycetes (e.g. *Trichoderma reesei*), basidiomycetes including white-rot fungi (e.g. *Phanerochaete chrysosporium),* brown-rot fungi and few anaerobic species which degrade cellulose in gastrointestinal tracts of ruminant animals - were so far investigated [65].
Fungi such as *T. reesei* and *A. niger* produce large amounts of free extracellular cellulolytic enzymes, whilst bacterial and few anaerobic fungal strains mostly produce cellulolytic enzymes assembled in a complex called cellulosome. As far as concern the basidiomycetes, they have unique oxidative systems that together with ligninolytic enzymes are responsible for lignocellulose degradation.
Among the basidiomycetes, one of the main studied strain is the white rot fungus *Pleurotus ostreatus*. Its genome has been fully sequenced [98], showing several genes coding for glycoside hydrolases (cellulases, xylanases, arabinofuranosidases, mannanases, pectinases), and ligninolytic enzymes (e.g. laccase), that find application in several industrial fields [99,100].

This chapter deals with the results concerning the development of new biosystems and biocatalysts for lignocellulosic biomass upgrading.
The following **section 2-I**, deals with the description of the activities carried out in the laboratory of Dr. Carlos Ricardo Soccol at the Department of “Engenharia de Bioprocessos e Biotecnologia” (DEBB) (Universidade Federal do Paraná, Brazil), in the frame of the project BIOASSORT. In detail, this section describes the results achieved starting from the selection of 32 lignocellulolytic fungal strains, belonging to the DEBB collection, for their ligninolytic, cellulolytic and xylanolytic activities production.
In the section 2-II, the paper “**Optimization of *Arundo donax* saccharification by (hemi)cellulolytic enzymes from *Pleurotus ostreatus***” describes the statistical analysis of the lignocellulosic biomass *Arundo donax* saccharification through the application of (hemi)cellulolytic enzymes produced by *Pleurotus ostreatus*, in order to identify the most significant parameters affecting the process.

2.2 Results

Section 2-I

2.2.1 Screening of fungal strains for cellulolytic, xylanolytic and ligninolytic activities production

2.2.1.1 Evaluation of enzymes production on solid medium

32 fungal strains from the DEBB Collection, belonging to the *Lentinus*, *Pleurotus* and *Aspergillus* spp, were analyzed for their cellulolytic and xylanolytic abilities by assessment of fungal growth and enzymes production on agar plates containing carboxymethylcellulose (CMC) or xylan as the sole carbon source. All the investigated strains were able to hydrolyze these substrates, showing to grow on them.

The screening was performed by a Congo red test, based on a formation of hydrolysis halo around the fungal colony, corresponding to the region of action of (hemi)cellulolytic enzymes; indeed, the Congo red dye only remains attached to regions where β-1,4-D-glucanohydrolase bonds were not hydrolysed [101]. According to Florencio et al. [102], the selection of strains that can efficiently degrade polysaccharides was carried out by evaluating the Enzymatic Index (EI), a ratio between the diameter of hydrolysis halo and the diameter of fungal mycelium. Fungal strains that showed an EI equal or higher than 1.4 were selected for the subsequent investigation of cellulases and xylanases production in liquid culture. Table 3 shows the EI of the investigated fungal strains after 5 days of incubation at 30 °C. The values given represent the average of the measurements of 2 experiments performed independently under the same conditions.

The strains *L. edodes* INRA L 465 (EI = 1.58), INRA 790 (EI = 1.4) and INRA 1022 (EI = 2.2) were selected for cellulase activity production, whilst the strains *Lentinus edodes* INRA 6635 (EI_{cell} = 1.5 and EI_{xyl} = 1.5), INRA 6647 (EI_{cell} = 1.4 and EI_{xyl} = 1.4) and INRA 6614 (EI_{cell} = 1.4 and EI_{xyl} = 1.4) and the strains *A. niger* NRRL 599 (EI_{cell} 1.4 and EI_{xyl} = 2.0), NRRL 2270 (EI_{cell} = 1.5 and EI_{xyl} = 2.6), NRRL 334 (EI_{cell} = 1.4 and EI_{xyl} = 1.4), NRRL 3312 (EI_{cell} = 1.4 and EI_{xyl} = 1.7), NRRL 1278 (EI_{cell} = 1.4 and EI_{xyl} = 2.0) and NRRL 511 (EI_{cell} = 1.5 and EI_{xyl} = 1.4) were selected for both cellulase and xylanase activities production. All these strains were, therefore, further analysed for a quantitative estimation of cellulase and xylanase production in liquid culture.

All the *Aspergillus*, *Pleurotus* and *Lentinus* strains were also investigated for their ligninolytic ability by using solid medium supplemented with the indicator Guaiacol. According to Ang et al. [103], the presence of ligninolytic activity was detected by observing the formation of a reddish brown halo around the fungal colonies due to the radial diffusion of the fungal enzyme able to oxidize guaiacol.

As reported in Table 3, among all the strains, *L. edodes* INRA 797, INRA 6608 and INRA 6646, *P. sajor-caju* INRA 31, INRA 3501 and INRA 3824 showed the highest level of Guaiacol oxidation and thus, they were further analysed for a quantitative estimation of laccase production in liquid culture.

Table 3. Detection of cellulolytic (A), xylanolytic (B) and ligninolytic (C) enzyme activities production on solid medium by 32 fungal strains belonging to the DEBB Collection

Fungal strains	**A)** EI for cellulase activity	**B)** EI for xylanase activity	**C)** Reaction level to Guaiacol *
Lentinus edodes INRA L 465 ▲**	1.58	1.1	±
Lentinus edodes INRA L 609	1.3	1.3	-
Lentinus edodes INRA 797 ●	1.2	1.2	+
Lentinus edodes INRA 790 ▲	1.4	ND	±
Lentinus edodes INRA 1022	2.2	1.2	-
Lentinus edodes INRA6602	ND	ND	-
Lentinus edodes INRA6608 ●	0.85	ND	+
Lentinus edodes INRA6609	ND	ND	-
Lentinus edodes INRA6613	1.3	1.25	-
Lentinus edodes INRA6121	ND	ND	-
Lentinus edodes INRA6624	ND	ND	-
Lentinus edodes INRA 6635 ▲ ■	1.5	1.5	±
Lentinus edodes INRA6646 ●	1.18	ND	+
Lentinus edodes INRA 6647 ▲ ■	1.4	1.4	±
Lentinus edodes INRA 6614 ▲ ■	1.4	1.4	±
Pleurotus sajor-caju INRA 31 ●	ND	ND	+
Pleurotus sajor-caju INRA 3501 ●	1.1	1.1	+
Pleurotus sajor-caju INRA 3824 ●	1.2	1.25	+
Aspergillus niger NRRL 599 ▲ ■	1.4	2.0	-
Aspergillus niger NRRL 2270 ▲ ■	1,5	2.65	-
Aspergillus niger NRRL 334 ▲ ■	1.4	1.4	-
Aspergillus niger NRRL 328	ND	ND	-
Aspergillus niger NRRL 3312 ▲ ■	1.4	1.76	-
Aspergillus niger NRRL 3	1.0	ND	-
Aspergillus niger NRRL 1278 ▲ ■	1.4	2.0	-
Aspergillus niger NRRL 511 ▲ ■	1.54	1.4	-
Aspergillus niger NRRL 2003	1.0	1.0	-
Aspergillus niger NRRL 2001	1.0	1.3	-
Aspergillus oryzae NRRL 1808	ND	ND	-
Aspergillus oryzae NRRL 3485	1.16	1.1	-
Aspergillus oryzae NRRL 2220	1.1	1.0	-
Aspergillus oryzae NRRL 1989	ND	1.1	-

* **+**: strong positive reaction; **-**: negative reaction; **±**: weak positive reaction.
ND: Not detected.

** Selected fungal strains for quantitative estimation of cellulolytic (▲), xylanolytic (■) and ligninolytic (●) enzyme activities in liquid culture

2.2.1.2 Evaluation of enzymes production in liquid medium

The 12 fungal strains selected for cellulases production on solid medium, taking into account the enzymatic index value, were subjected to a quantitative evaluation of enzymes production in liquid culture containing microcrystalline cellulose as carbon source. Among the investigated strains, the screening led to the selection of the strain *L. edodes* INRA L 465, showing a maximum of cellulase activity production of 0.23 U/mL after 12 days of fermentation (Fig. 6) and, the strains *A. niger* NRRL 3312 and *A. niger* NRRL 511 showing 0.28 U/mL of cellulase activity after 14 days of fermentation (Fig. 7).

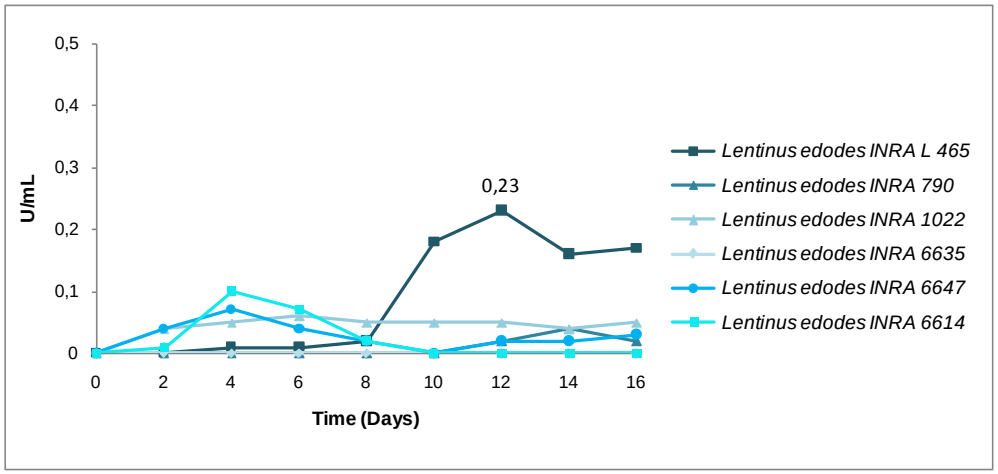


Figure 6: Kinetics of cellulase production in liquid culture by *Lentinus edodes* strains. Standard deviation is less than 5%

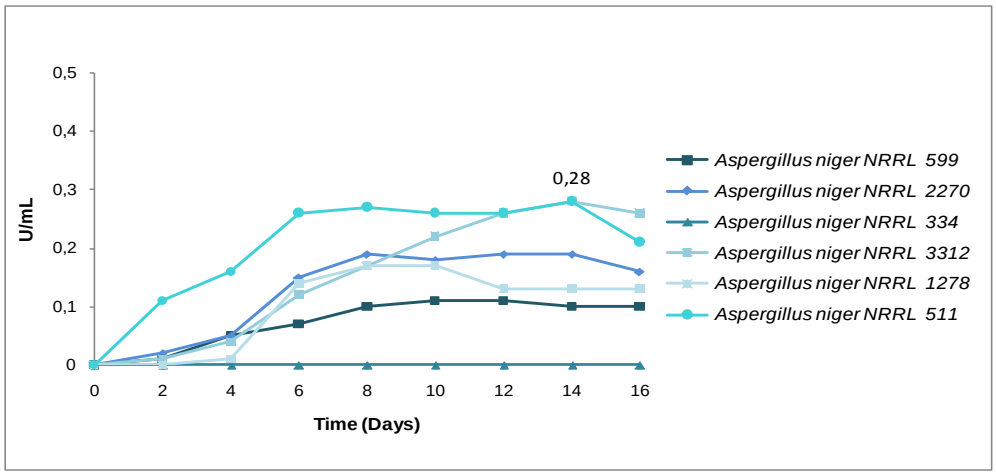


Figure 7: Kinetics of cellulase production in liquid culture by *Aspergillus niger* strains. Standard deviation is less than 5%

As far as concern the 9 strains selected for their xylanases production on solid medium, the time course of enzymatic activity production in liquid culture containing microcrystalline cellulose as carbon source led to the selection of the strain *Aspergillus niger* NRRL 3312, showing a maximum xylanase activity of 19.18 U/mL

after 16 days (Fig. 8). Further incubation did not show any increment in the level of enzyme production, probably due to depletion of nutrients in the media.
The laccases activity production in liquid culture of *Lentinus edodes* INRA 797, INRA 6608, INRA 6646 and *Pleurotus sajur-caju* INRA 31, INRA 3501, INRA 3824 grown at 30°C with copper sulfate as inducer, is shown in Fig. 9. Among them, the best producer of laccases was *Pleurotus sajur-caju* INRA 31 with a maximum value of 12.07 U/mL after 12 days.

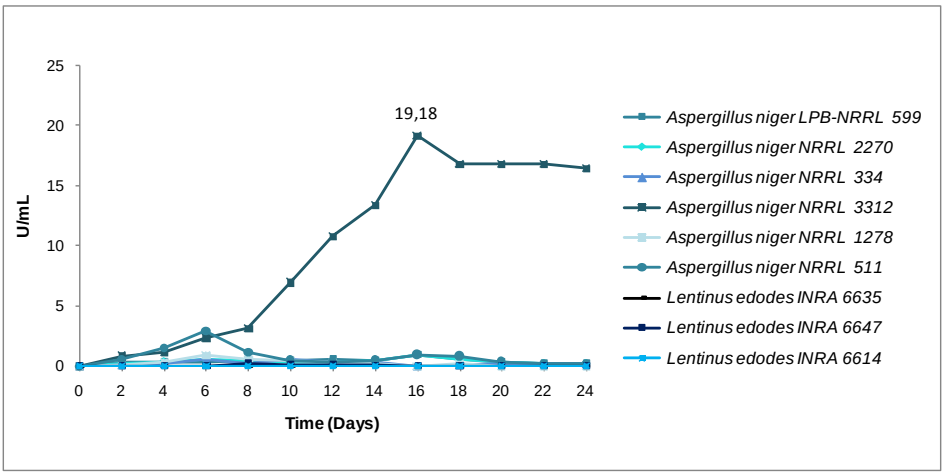


Figure 8: Kinetics of xylanases production in liquid culture by *Aspergillus niger* and *Lentinus edodes* strains. Standard deviation is less than 10%

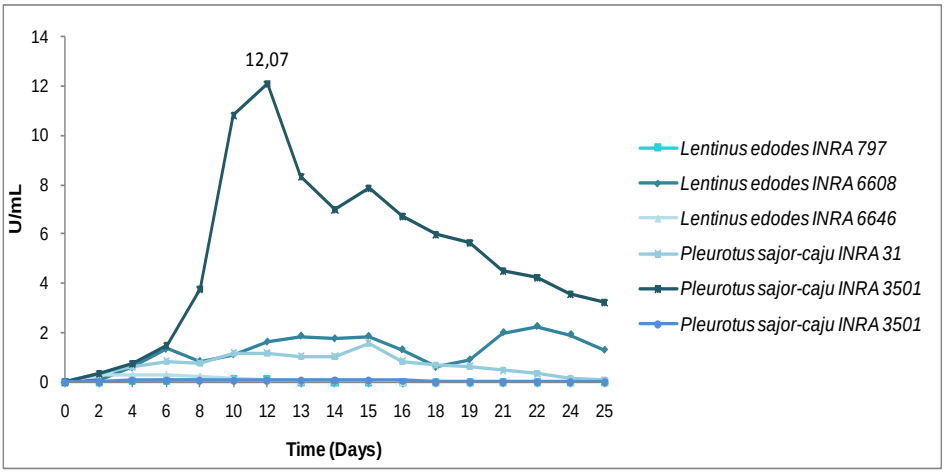


Figure 9: Kinetics of laccases production in liquid culture by *Pleurotus sajor-caju* and *Lentinus edodes* strains. Standard deviation is less than 10%

2.2.1.3 Evaluation of enzymes production by solid state fermentation on BSG - Cellulases and xylanases production by *Aspergillus niger* NRRL 3312

The strain *Aspergillus niger* NRRL 3312 demonstrated its ability to colonize the BSG quickly and extensively. The time course of cellulases activities production revealed a maximum value of 118.04 U g^{-1} of dry substrate (gds) after 10 days of fermentation

(Fig. 10 A), while concerning the xylanases production, a maximum of 1315.15+ U gds^{-1} of dry substrate was reached after 4 days of fermentation (Fig. 10 B).

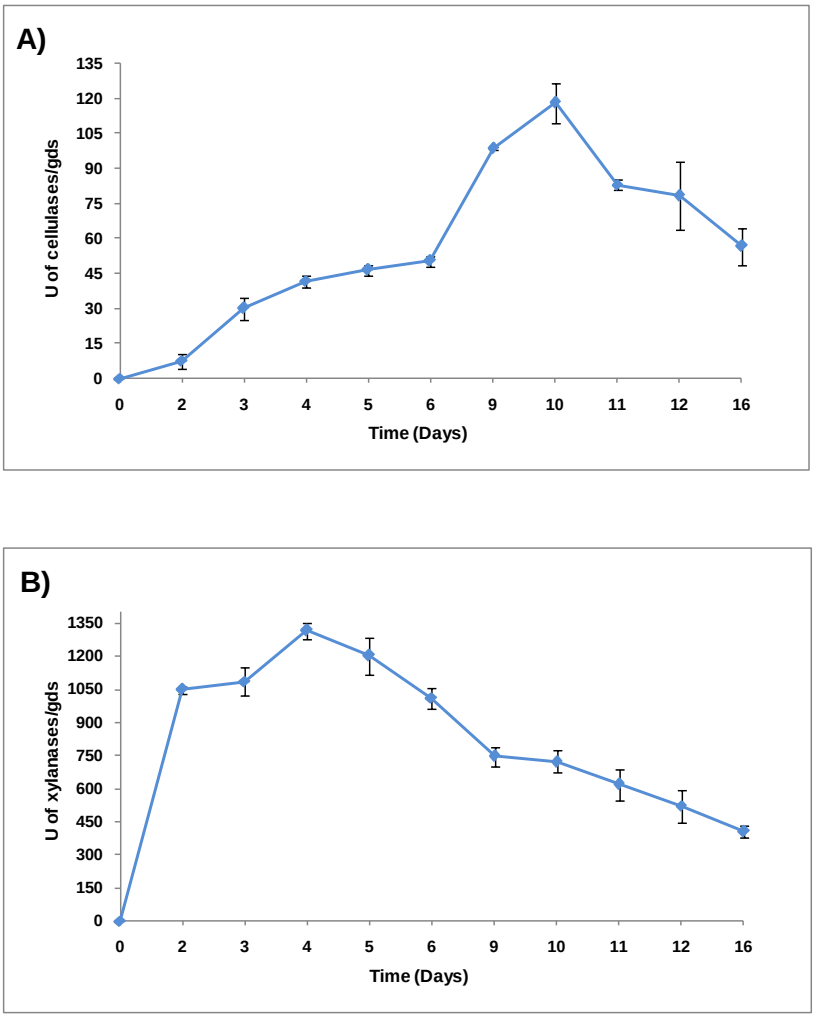


Figure 10: Time course of **A)** cellulase and **B)** xylanase activities produced by *Aspergillus niger* NRRL 3312 in SSF on BSG

The co-production at higher levels of cellulases and xylanases enzymes was an unusual behaviour for the *Aspergillus* spp, as proved by few works so far reported in literature [104,105,106,107]. When compared with the xylanases production by SSF reported for other *Aspergillus spp.*, the maximum value achieved by *A.* niger NRRL 3312 resulted mostly higher [108,109,110] and only in very few cases, lower [104,105] (Table 4).
Regarding the cellulases production, the maximum value achieved by *A.* niger NRRL 3312 was, in some cases, higher [111,112,113] and in some others lower [104, 105] than those reported for other *Aspergillus* strains (Table 4). Since no optimization was performed in our study, this process presents good perspective for high cellulase and xylanase production.

In order to identify the putative proteins responsible for cellulase and xylanase activities of *A. niger* NRRL 3312, zymogram and proteomics analyses on the samples that showed the maximum activities production (4° and 10° days of SSF for xylanase and cellulase, respectively) were performed. One activity halos for cellulase and two activity halos for xylanase were visualized, and in correspondence to these halos, different bands were detected on the gel stained with Blue comassie (Figure 12A-12B). Regarding the cellulases, three comassie stained bands, corresponding to the activity halo were analyzed (Table 5).

Table 4: Comparison of cellulases and xylanases production by *Aspergillus* spp

Microorganism	Substrate	Maximum xylanase activity	Maximum cellulase activity	References
A. niger KK2	Rice straw	5070 U gds^{-1}	19.5 U gds^{-1} FPase 129 U gds^{-1} CMCase, 100 U gds^{-1} β-glucosidase	[104]
A. niger USM AI 1	Sugarcane bagasse and palm kernel cake	-	3.4 U/ gds FPU	[111]
A. niger from Dharmapuri Dist, Tamil Nadu, India	Coir waste	-	8.89 U gds^{-1} CMCase, 3.56 U gds^{-1} FPase activities	[112]
A. niger JL-15	Orange peel	917.7 U gds^{-1}	-	[108]
A. niger NS-2	Wheat bran	-	17 U gds^{-1} CMCase, 310 U gds^{-1} FPase 33 U gds^{-1} β-glucosidase	[113]
A. niger FGSCA733	*Jatropha curcas* seed cake	6087 U gds^{-1}	3974 U gds^{-1}	[105]
A. niger NRRL 328	Soybean Husks	950 U gds^{-1}	-	[109]
A. fumigatus SK1	Untreated oil palm trunk	418.7 U gds^{-1}	54.27 U gds^{-1} of CMCase, 3.36 U gds^{-1} of FPase and 4.54 U gds^{-1} of β-glucosidase	[106]
A. fumigatus P40M2 and *A. niger* P47C3	wheat bran	1055.6 U gds^{-1} 1258.0 U gds^{-1}	105.8 U gds^{-1} of β-glucosidase 96.0 U gds^{-1} of β - glucosidase	[107]
A. niger F-3	Citrus peels	250 U gds^{-1}	-	[110]

Proteomic analysis confidently assessed the presence, in the first band, of 8 peptides matching to peptides present in the sequence of a 1,4-β-D-glucan cellobiohydrolase B (gi: 4698498), 5 peptides matching to peptides present in the sequence of a 1,4-β-D-glucan cellobiohydrolase C [*Aspergillus niger* CBS 513.88] (gi: 145246118), 4 peptides matching to peptides present in the sequence of a endo-β-1,4-glucanase B [*Aspergillus niger* CBS 513.88] (gi: 145238644), and 4 peptides matching to peptides present in the sequence of a endoglucanase A [*Aspergillus niger*] (gi: 3757552); in the second band were found 10 peptides matching to peptides present in the sequence of a 1,4-β-D-glucan cellobiohydrolase A (gi: 74698499) and 5 peptides matching to peptides present in the sequence of a 1,4-β-D-glucan cellobiohydrolase C [*Aspergillus niger* CBS 513.88] (gi: 145246118); in the third band were found 13 peptides matching to peptides present in the sequence of a endo-β-1,4-glucanase B [*Aspergillus niger* CB] (gi: 145238644) and 6 peptides matching to peptides present in the sequence of a endo-β-1,4-glucanase A [*Aspergillus niger* CBS 513.88] (gi: 145230537).
Concerning the xylanases, proteomic analysis confidently assessed the presence of 3 peptides matching to peptides present in the sequence of a endo-1,4-β-xylanase A OS=*Aspergillus kawachii* (gi: 380865430) in the first band, the presence of 5 peptides matching to peptides present in the sequence of a exo-1,4-β-xylosidase xlnD OS=*Aspergillus awamori* (gi: 121809149) in the second band and the presence of 2 peptides matching to peptides present in the sequence of a endo-1,4-β-xylanase B OS=*Aspergillus kawachii* (gi: 380865431); unlike, for the third band, no peptides matching with protein responsible for xylanase activity were found (Table 6).

Table 5: LC/MS-MS analysis results of the 3 selected bands corresponding to the cellulase halo activity from *Aspergillus niger* NRRL 3312

Band	NCBInr ID	Protein	Score	N° of peptides	Sequence coverage
B1	4698498	1,4-β-D-glucan cellobiohydrolase B	409	8	25 %
	145246118	1,4-β-D-glucan cellobiohydrolase C [*Aspergillus niger* CBS 513.88]	222	5	35 %
	145238644	endo-β-1,4-glucanase B [*Aspergillus niger* CBS 513.88]	297	4	14 %
	3757552	endoglucanase A [*Aspergillus niger*]	194	4	20 %
B2	74698499	1,4-β-D-glucan cellobiohydrolase A	673	10	38 %
	145246118	1,4-β-D-glucan cellobiohydrolase C [*Aspergillus niger* CBS 513.88]	219	5	27 %
B3	145238644	Endo-β-1,4-glucanase B [*Aspergillus niger* CB]	828	13	47 %
	145230537	Endo-β-1,4-glucanase A [*Aspergillus niger* CBS 513.88]	470	6	14 %

Table 6: LC/MS-MS analysis results of the 4 selected bands corresponding to the xylanase halos activity from *Aspergillus niger* NRRL 3312

Band	NCBInr ID	Protein	Score	N° of peptides	Sequence coverage
B1	XYNA_ASPKW	Endo-1,4-β-xylanase A OS=*Aspergillus kawachii*	129	3	13 %
B2	XYND_ASPAW	Exo-1,4-β-xylosidase xlnD OS=*Aspergillus awamori*	141	5	7 %
B3	-	-	-	-	-
B4	XYNB_ASPKW	Endo-1,4-β-xylanase B OS=*Aspergillus kawachii*	105	2	14 %

- Cellulases production by *Lentinus edodes* INRA L 465

The experiments of SSF with *L. edodes* INRA L 465 revealed the incapacity of the fungus to colonize the waste quickly and extensively. Indeed, more than one month was necessary to allow the initial fungal colonization, causing the deterioration of the substrate. Therefore, no further experiments were carried out.

- Laccases production by *Pleurotus sajor-caju* INRA 3501

Pleurotus sajor-caju INRA 3501 was able to colonize the BSG extensively. Concerning the laccases production, the strain showed a maximum value of 157.36 U gds^{-1} after four days of fermentation (Fig. 11). In comparison with other SSF processes previously reported by using *P. sajor-caju* [114,115] and other *Pleurotus spp.* [116,117] (Table 7), the strain *P. sajor-caju* INRA 3501 allowed to achieve higher values of laccases activity, Even without performing culture conditions optimization.

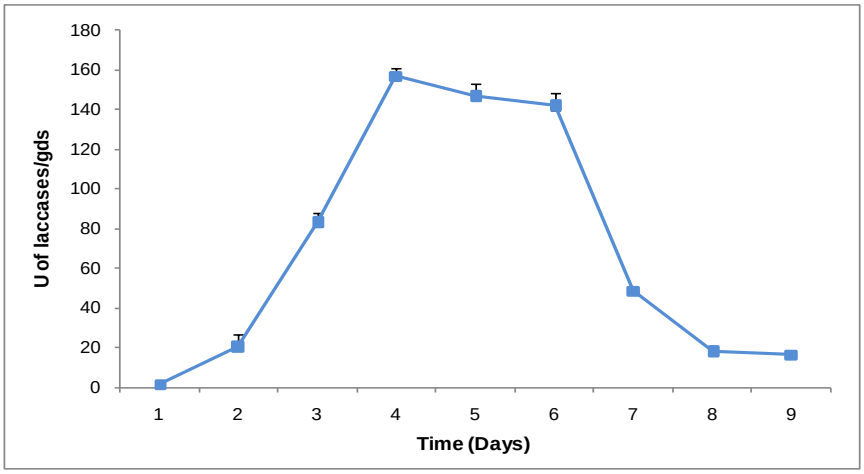


Figure 11: Time course of laccases activity produced by *Pleurotus sajor-caju* INRA 3501 in SSF on BSG

In order to identify proteins putatively responsible for laccase activity of *P. sajor-caju* INRA 3501, zymogram and proteomics analyses on the sample that showed the maximum activity production (4° days of SSF) were performed. Three activity halos for laccase were visualized, and in correspondence to these three halos, different bands were detected on the gel stained by Blu commassie (Figure 12C). Proteomic

analysis confidently assessed the presence, in the first band, of the 5 peptides matching to peptides present in the sequence of a laccase I from *Trametes versicolor* FP-101664 SS1(gi: 392570979) and 4 peptides matching to peptides present in the sequence of a laccase 3 precursor from *Trametes villosa* (gi: 7432992). Unlike, for the remaining four bands, no peptides matching with protein responsible for laccase activity were found (Table 8).

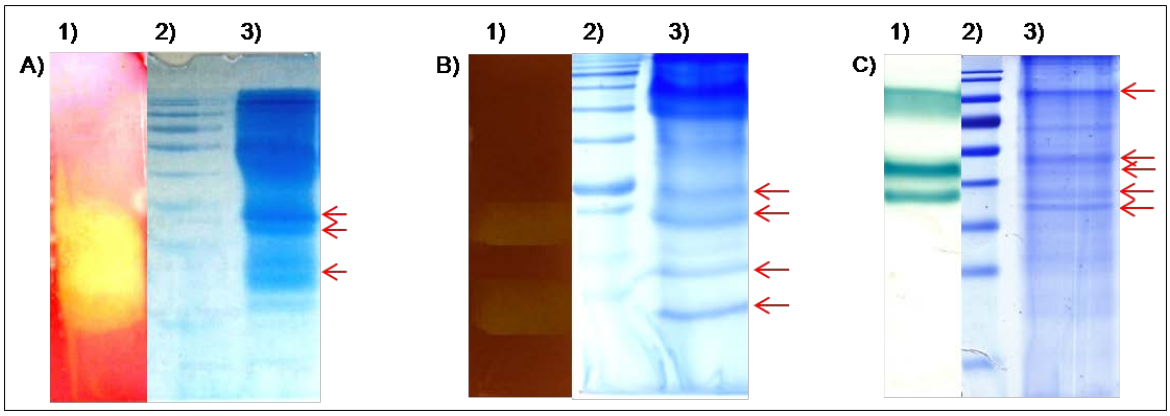


Figure 12: Analyses of supernatant of *Aspergillus niger* NRRL 3312 strain. **A_1)** Zymogram analysis for cellulase detection on agar plate with 1 % CMC stained with Congo Red; **B_1)** Zymogram analysis for xylanase detection on agar plate with 1 % xylan stained with Congo Red. Analyses of supernatant of *Pleurotus sajor-caju* INRA 3501. **C_1)** Zymogram analysis for laccase detection by submerging the gel with ABTS 20 mM in sodium citrate buffer (0.1 M, pH 3.0) after electrophoresis; **A-B-C_{2-3})** SDS-PAGE (12.5 %) without denaturating and reducing treatments stained with Blue Commassie. **lane 2**: protein molecular weight marker; **lane 3:** 80µg of total protein

Table 7: Comparison of laccase activity values reported for *Pleurotus* spp

Microorganism	Substrate	Maximum laccase activity	References
P. sajor-caju	Sago *hampas*	17.7 U gds^{-1}	[115]
P. ostreatus and P. sajor-caju	Different agro-wastes	5.48 U/mg of protein on (2:1) wheat straw:bran 3.85 U/mg of protein on (2:1) sesame straw:bran	[114]
P. florida NCIM 1243	Banana peel : mandarin peel : cantaloupe peel in a ratio of 5:2:3	5.4 U/gds	[116]
P. ostreatus	Wheat bran	32.45 U/gds	[117]

Table 8: Data by analysis LC/MS-MS analysis results of the 5 selected bands corresponding to the laccase halos activity from *Pleurotus sajor-caju* INRA 3501

Band	NCBInr ID	Protein	Score	N° of peptides	Sequence coverage
B1	392570979	Laccase I [*Trametes versicolor* FP-101664 SS1]	145	5	13 %
	7432992	Laccase (EC 1.10.3.2) 3 precursor - white-rot fungus [*Trametes villosa*]	127	4	12 %
B2	-	-	-	-	-
B3	-	-	-	-	-
B4	-	-	-	-	-
B5	-	-	-	-	-

2.2.1.4 Materials and Methods

- Screening for cellulases, xylanases and laccases production on agar medium.

All the fungal strains (*Pleurotus* spp, *Lentinus* spp and *Aspergillus* spp) listed in Table 3 were grown on potato dextrose yeast agar (PDY) plate [composition (g/L): potato dextrose (24.0), yeast nitrogen base (5.0), agar powder (15.0)] for 5 days at 30°C. In order to evaluate their cellulases and xylanases production, agar cores (1,3 mm in diameter) from 6-days old agar cultures were transferred on PDY agar plates containing 1 % carboxylmethylcellulose (CMC) and 1 % xylan, respectively. After a 6 days incubation , the strains were assayed for their ability to degrade CMC/Xylan by incubation with 0.1 % Congo Red solution for 30 min, followed by washing step with 5 M NaCl solution as reported in Amore et al. [118]. The selection of strains that can efficiently degrade polysaccharides was carried out according to Florencio et al. [102].

To detect ligninolitic activity, a PDY medium containing the oxidation indicator Guaiacol at a concentration of 0.01 % (w/v) was used. The strains that showed the highest levels of Guaiacol oxidation were considered to be potential producers of laccases and were selected for further analysis.

- Screening for cellulases, xylanases and laccases production in liquid culture

In order to perform a quantitative estimation of enzymatic activities production, two different media were chosen: medium **A** [composition (g/L): yeast extract (0.5); $MgSO_4 \cdot 7H_2O$ (0.3); $FeSO_4 \cdot 7H_2O$ (0.005); $MnSO_4 \cdot H_2O$ (0.00156); $ZnSO_4 \cdot 7H_2O$ (0.0014); $CaCl_2$ (0.3); $CoCl_2$ (0.002) and KH_2PO_4 (1.5) at pH 5.5, supplemented with microcrystalline cellulose (10.0)] for cellulases and xylanases production analysis, and medium **B** [composition (g/L): glucose (10.0); yeast extract (0.5); $MgSO_4 \cdot 7H_2O$ (0.3); $FeSO_4 \cdot 7H_2O$ (0.005); $MnSO_4 \cdot H_2O$ (0.00156); $ZnSO_4 \cdot 7H_2O$ (0.0014); $CaCl_2$ (0.3); $CoCl_2$ (0.002) and KH_2PO_4 (1.5) at pH 5.5, supplemented with 150 µM copper sulfate at the time of inoculation] for laccases production analysis.

As far as concern *Lentinus* spp and *Pleurotus* spp, 3 agar cores (1,3 mm in diameter) from 6-days old agar cultures were pre-inoculated into 50 mL of PDY medium and, after 6 days, the inoculum at 10 % in medium A or B were carried out.

Regarding *Aspergillus* spp, spore suspension obtained by mixing pre-cultured fungus, were inoculated in medium A or B at a final concentration of 10^7 spores g^{-1} of carbon source.

All the flasks were incubated at 30°C, 120 rpm and sampling for enzymatic quantification were performed every day for 25 days.

- Solid state fermentation on Brewer's spent grain

Pre-inoculum of *Aspergillus* spp and spore suspension were performed as reported in Montibeller et al. [109]. Approximately 10^7 spores g^{-1} were inoculated on 25 g of dry Brewer's spent grain, in 250-mL Erlenmeyer' s flasks, in duplicate. The initial moisture were adjusted at 70 % (w/v) by adding enough volume of distilled water and a mineral salt solution containing (g/L): KH_2PO_4 (1.5); $CuSO_4$ (0.4) and $CoSO_4$ (0.0012).

Concernig *Lentinus* spp and *Pleurotus* spp, pre-inoculum and inoculum was carried out as described in Iandolo et al. [119]

The flasks were incubated statically at 30 °C for 16 days. Enzymes extraction was performed according to Montibeller et al. [109].

- Enzyme assays

CMCase and xylanase activity assays were carried out according to Ghose [120] and Bailey et al. [121], respectively. Released reducing sugars were determined by dinitrosalicylic acid reagent (DNS) method [122].

Laccase activity was assayed at 25 °C, using 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (ABTS) as substrate as previously described [123].

- Zymogram analyses and protein identification by mass spectrometry

Semi-denaturing gel electrophoresis (SDS-PAGE) was performed by loading non-denatured and not-reduced samples on a SDS polyacrylamide gel, as described by Laemmli [124]. Proteins showing cellulolytic and xylanolytic activities were visualized according to Amore et al. [125,126], respectively, while proteins showing ligninolytic activity were visualized as described by Karp et al. [127].

Slices showing the activities of interest were cut from the SDS-PAGE and analysed by mass spectrometry as reported in Amore et al. [125].

Section 2-II

2.3 "Optimization of *Arundo donax* saccharification by (hemi)cellulolytic enzymes from *Pleurotus ostreatus*" (Paper I)

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Research Article

Optimization of *Arundo donax* Saccharification by (Hemi)cellulolytic Enzymes from *Pleurotus ostreatus*

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An enzymatic mixture of cellulases and xylanases was produced by *Pleurotus ostreatus* using microcrystalline cellulose as inducer, partially characterized and tested in the statistical analysis of *Arundo donax* bioconversion. The Plackett-Burman screening design was applied to identify the most significant parameters for the enzymatic hydrolysis of pretreated *A. donax*. As the most significant influence during the enzymatic hydrolysis of *A. donax* was exercised by the temperature (°C), pH, and time, the combined effect of these factors in the bioconversion by *P. ostreatus* cellulase and xylanase was analyzed by a 3^3 factorial experimental design. It is worth noting that the best result of 480.10 mg of sugars/gds, obtained at 45°C, pH 3.5, and 96 hours of incubation, was significant also when compared with the results previously reached by process optimization with commercial enzymes.

1. Introduction

To solve the issues related to the petroleum-based energy and compounds, more attention has been focused on the use of lignocellulosic wastes as source for added-value bioproducts [1]. They show several advantages such as low cost and world-wide availability with an equal geographical distribution [2]. The major component of lignocellulosic materials is cellulose that is present in the cell wall within a matrix of hemicellulose and lignin bonded by cross-linkages. This complex structure requires mainly three steps for the conversion of lignocelluloses into added-valued bioproducts: (i) a pretreatment to remove lignin and expose the polysaccharides, (ii) hydrolysis of polysaccharides that can be performed enzymatically using an enzymatic cocktail composed of cellulases and hemicellulases, and (iii) fermentation of the sugars into the desired bioproducts.

The enzymatic hydrolysis represents the limiting step of the overall process due to the high costs of the employed enzymes cellulases, a group of enzymes comprising cellobiohydrolase (CBH), *endo*-1,4-β-D-glucanase (EG), and β-glucosidase (BG), and hemicellulases, including xylanase, xyloglucanase, mannanase, arabinase, galactanase, polygalacturonase, glucuronidase, acetyl xylan esterase, and other enzymes [3]. Extensive studies were reported in the last few decades in order to identify new more efficient enzymes [4–7] and to obtain a high yield of sugars with low enzymes dosage [8].

Filamentous fungi represent the major source of cellulases and hemicellulases and are able to produce large amounts of lignocellulosic enzymes in different growth conditions [9].

In this study, the basidiomycetous fungus *Pleurotus ostreatus* was employed as a source of (hemi)cellulolytic enzymes that were partially characterized and applied to the hydrolysis of the lignocellulosic biomass *Arundo donax*. This biomass was chosen since it can be cultivated on lands such as hilly areas that are considered not appropriate for the traditional cereal production because yield is low and because

the traditional cropping system causes extreme vulnerability to soil erosion [10]. In these conditions, perennial biomass crops such as giant reed (*Arundo donax* L.) proved to reduce soil erosion and to increase potential gross income of farmers [11] with favourable environmental impacts [12]. This allows avoiding competition with the use of lands for food production.

In order to optimize the application of *P. ostreatus* (hemi)cellulolytic enzymes to *A. donax* hydrolysis, statistical analysis of biomass conversion by the investigated fungal enzymatic cocktail was performed. To identify the most significant parameters for the enzymatic hydrolysis, the Plackett-Burman screening design was applied and the combined effect of the most significant factors identified (temperature (°C), pH, and time) was analyzed by a 3^3 factorial experimental design.

2. Materials and Methods

2.1. Microorganism. The strain *Pleurotus ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC number MYA-2306) was maintained through periodic transfer at 4°C on solid medium containing 15 g/L agar and PDY [24 g/L potato dextrose (Difco, Detroit, Michigan, USA) and 5 g/L yeast extract (Difco)].

2.2. Preinoculum. Precultures were prepared by inoculating 500 mL of PDY broth in 1 L Erlenmeyer flask with six agar plugs (⌀ = 11 mm) of *P. ostreatus* mycelium, from the edge of a 7-day-old agar culture, in a temperature-controlled incubator at 28°C on a rotary shaker at 120 rpm for six days. After homogenizing through sterile blender, the mycelia were washed with sterile distilled water three times under laminar flow cabinet. The washed mycelia were inoculated (10% v/v) in the medium A with the following composition: $MgSO_4 \cdot 7H_2O$ (0.3 g/L), $FeSO_4 \cdot 7H_2O$ (0.005 g/L), $MnSO_4 \cdot H_2O$ (0.00156 g/L), $ZnSO_4 \cdot 7H_2O$ (0.0014 g/L), $CaCl_2$ (0.3 g/L), $CoCl_2$ (0.002 g/L), yeast extract (0.5 g/L), KH_2PO_4 (1.5 g/L), and pH 5.5.

2.3. Analysis of Inducers of P. ostreatus on Cellulase and Xylanase Activities Production. Preliminary experiments were carried out in 24-well plate flat bottom with Low Evaporation Lid (BD-Falcon, Franklin Lanes, New Jersey, USA) containing 1.5 mL of medium A and 10% v/v of homogenized mycelia of *P. ostreatus* in each well. The medium was supplemented with different carbon sources: xylan from beachwood (Sigma-Aldrich, St. Louis, MO, USA), carboxymethylcellulose (CMC), sodium salt medium viscosity (Sigma-Aldrich, St. Louis, MO, USA), 99% xylitol (Alfa Aesar, Parkridge Road, Ward Hill, MA, USA), D-(+)- 98% cellobiose (Alfa Aesar, Parkridge Road, Ward Hill, MA, USA), L(+)-Arabinose (Merck Millipore, Darmstadt, Germany), 98% L-(−)-Arabitol (Alfa Aesar, Parkridge Road, Ward Hill, MA, USA), microcrystalline cellulose (Alfa Aesar, Parkridge Road, Ward Hill, MA, USA), sophorose 0.6 mM (Sigma-Aldrich, St. Louis, MO, USA), D(+)-Xylose (Sigma-Aldrich, St. Louis, MO, USA), Arabinan (Megazyme), wheat arabinoxylan low viscosity (Megazyme), D-(+)Galactose (Sigma-Aldrich, St. Louis, MO, USA), and lactose (Carlo Erba, Milan, Italy), tested at final concentration of 1% (w/v), with the exception of the sophorose, tested at final concentration of 0.6 mM. The plates were incubated at 28°C on a rotary shaker at 250 rpm for 14 days. Samples were centrifuged at 13.000 rpm for 15 minutes and the supernatants were used for cellulase and xylanase assays.

The selected carbon sources were further investigated for their inductive effect at final concentration of 1% (w/v) in 1 L Erlenmeyer flask, containing 500 mL of medium and 10% v/v of homogenized *P. ostreatus* mycelia. The flasks were incubated at 28°C on a rotary shaker at 120 rpm for 35 days. Samples were collected for cellulase and xylanase assays as described above.

2.4. Enzymatic Activity Assays

2.4.1. Xylanase Assay. Xylanase activity assay was performed according to Bailey et al. [13].

2.4.2. Azo-CMCase Assay for Endo-1,4-β-Glucanase. Endo-1,4-β-Glucanase activity produced in liquid culture was assayed by using Azo-CMC (Megazyme, Ireland) as substrate, following supplier's instructions.

2.4.3. Dinitrosalicylic Acid Assay for Endo-1,4-β-Glucanase. For assessing the optimum pH and temperature and thermal- and pH-resistance, endo-1,4-β-Glucanase activity was assayed towards CMC (Sigma-Aldrich, St. Louis, MO, USA) as substrate, following the DNS assay method reported by Ghose [14].

2.4.4. β-Xylosidase, β-Glucosidase, and α-Arabinofuranosidase Assays. β-Xylosidase, α-arabinofuranosidase, and β-glucosidase activities were determined by using *p*-nitrophenyl-glycoside substrates as described in Marcolongo et al. [15]. All the enzymatic measurements were performed in triplicate.

2.5. Optimum Temperature and Thermoresistance. Supernatant of *P. ostreatus* was concentrated by ultrafiltration with a 10 kDa polyethersulfone membrane (Millipore Corporation, Bedford, MA, USA) and subjected to the determination of optimum temperature and thermoresistance of the xylanase and cellulase.

To assess the optimum temperature, the substrates birchwood xylan (Sigma-Aldrich, St. Louis, MO, USA) and CMC (Sigma-Aldrich, St. Louis, MO, USA) used for the xylanase and cellulase activities assays, respectively, were dissolved in 50 mM Na citrate at pH 5.3 and the incubations in presence with the enzymatic preparation were performed at 30, 40, 50, 60, 70, and 80°C. The thermoresistance of the xylanase and cellulase activities was investigated by incubating the fungal culture supernatant in 50 mM Na citrate pH 5.3, at 30°C, 40°C, and 50°C.

The reported results correspond to mean values of the three independent experiments, each one performed in three replicates.

2.6. Optimum pH and pH Resistance. The optimum pH of *P. ostreatus* cellulase and xylanase was determined on the supernatant of fungal culture concentrated by ultrafiltration with a 10 kDa polyethersulfone membrane (Millipore Corporation, Bedford, MA, USA). The experiments were performed at 25°C using both McIlvaine buffer, with pH values between 3.0 and 9.0, and Na-citrate buffer, at pH ranging from 3.0 to 6.0, performing the cellulase and xylanase activities assays with the substrates CMC and birch-wood xylan, respectively, dissolved in the above-mentioned buffers.

The pH resistance of the cellulase and xylanase activities was analyzed by diluting the supernatant in McIlvaine buffer, with pH ranging from 3.0 to 9.0, and incubating at 25°C.

The reported results correspond to mean values of the three independent experiments each one performed in three replicates.

2.7. Enzymatic Hydrolysis. The enzymatic hydrolysis mixtures, set up according to the experimental designs, contained the pretreated *A. donax* biomass previously oven-dried at 50°C to a moisture content less than 10% (w/w) in a total volume of 2 mL consisting of 50 mM sodium citrate buffer plus the enzyme cocktail. The biomass hydrolysis was carried out with the following enzymatic preparations, whose units per grams of dry pretreated substrate are specified below in the following paragraph on Plackett-Burman (PB) design: cellulase and xylanase activities from *P. ostreatus* after 9 days of growth and the cellulase from *Trichoderma reesei* ATCC26921. The commercial enzymatic mix C (145 U/gds of cellobiase from *A. niger* and 8 U/gds of thermostable β-xylosidase) was also included in the reaction mix to obtain the complete carbohydrates hydrolysis into the respective monomers. The hydrolysis mixtures, supplemented with 40 g/mL tetracycline and 30 g/mL cycloheximide to prevent microbial contamination, were prepared in caped tubes and were incubated together with blanks (pretreated lignocellulosic material without enzyme cocktail) on a rotary thermoblock (Thermomixer C, from Eppendorf) at 600 rpm. Different hydrolysis conditions were tested according to either "Plackett-Burman (PB) design" or "3^3 factorial experimental designs." Samples were withdrawn at different time intervals, chilled on ice, and centrifuged at 16.500 ×g for 30 min at 4°C.

The total released sugars were expressed as the amount (mg) of total soluble sugars liberated after hydrolysis per grams of pretreated biomass.

2.8. Determination of Sugar Content. The sugars contained in the cleared supernatants obtained from *A. donax*, pretreated and subjected to the two-step acid hydrolysis or the enzymatic hydrolysis as described above, were analyzed by a high-performance liquid chromatographic (HPLC) system (Dionex, Sunnyvale, CA, USA), equipped with an anionic exchange column (Carbopac PA-100) and a pulsed electrochemical detector. Glucose and xylose were separated with 16 mM sodium hydroxide at a flow rate of 0.25 mL/min and identified by the respective standards. Fucose was used as internal standard.

2.9. Experimental Design and Data Analysis. In order to elucidate the most significant conditions for the enzymatic hydrolysis of pretreated *A. donax* the Surface Response Methodology (SRM) was used. The Plackett-Burman (PB) factorial design, used to identify the critical parameters, and the 3^3 factorial experimental design were obtained by the Statistica 12.0 software (Statsoft Inc., 2013). The regression coefficients, analysis of variance (ANOVA), and p and F values were used to estimate the statistical parameters employed by the same software. ANOVA table consists of calculations that provide information about levels of variability within a regression model and form a basis for tests of significance.

2.10. Plackett-Burman (PB) Design. A total of 11 (N) variables including temperature (°C), amount of biomass (%, w/v), pH, time (hours), concentration of cellulase and xylanase from *P. ostreatus* and of commercial cellulase from *Trichoderma reesei* ATCC26921 (U/gds), and 5 unassigned variables (dummy) were studied in 12 ($N + 1$) experiments. Each variable was examined at two levels, high and low, denoted by (+1) and (−1) signs, respectively (Table S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2015/951871). Also 3 centre points, in which the medium level with the code (0) was considered for each parameter, were included in the PB matrix (Table 1). The main effect of each variable was determined using the following equation:

$$E_{xi} = \frac{\left(\sum M_{i+} - \sum M_{i-}\right)}{N}, \quad (1)$$

where E_{xi} is the variable main effect and M_{i+} and M_{i-} are the sum of the responses in runs, in which the independent variable (xi) was present in high and low levels, respectively, while N is the half number of runs considered. If the main effect of the tested variable is positive, it means that the influence of the concerning variable is greater at the high level tested, and when it is negative, the influence of the given variable is greater at the low level.

2.11. 3^3 Factorial Experimental Design. To investigate the effect of the most critical parameters, defined through the PB factorial design, as well as how their interactions affect the response variable, 3^3 factorial experimental design was performed (Table S2). According to the 3^3 full factorial design for the three variables, 27 experimental runs including 3 central points, totalizing 30 runs were executed (Table 2).

2.12. Validation of Developed Model. Based on the 3^3 factorial experimental results, complementary experiments (in triplicate) were carried out to validate the developed model. The experiment performed incorporated the conditions (run number 18) that allowed obtaining the maximum amount of released sugars. After, the experimental responses obtained were compared to the theoretical responses calculated from the developed model. The difference between the experimental and theoretical responses was evaluated to test the reliability of the model to predict the yield of saccharification of *A. donax*.

Table 1: Plackett-Burman 11/12 screening design: two levels, 6 factors, 3 central points, and 5 dummy factors (D1, D2, D3, D4, and D5).

Run	Temperature (°C)	% biomass (w/v)	pH	Cellulase from PO (U/gds)[a]	Cellulase from *Trichoderma reesei* ATCC26921 (U/gds)	Time (hours)	Dummy 1	Dummy 2	Dummy 3	Dummy 4	Dummy 5	Total sugars released (mg/gds)
1	45	7	3	10	1.8	24	1	1	1	−1	1	179.15
2	45	3	6	30	1.8	24	−1	1	1	1	−1	509.95
3	25	7	6	10	5.4	24	−1	−1	1	1	1	173.70
4	45	7	3	30	1.8	72	−1	−1	−1	1	1	408.55
5	45	3	6	30	5.4	24	1	−1	−1	−1	1	439.30
6	45	7	6	10	5.4	72	−1	1	−1	−1	−1	312.75
7	25	7	6	30	1.8	72	1	−1	1	−1	−1	112.15
8	25	7	3	30	5.4	24	1	1	−1	1	−1	344.35
9	25	3	3	30	5.4	72	−1	1	1	−1	1	461.70
10	45	3	3	10	5.4	72	1	−1	1	1	−1	598.30
11	25	3	6	10	1.8	72	1	1	−1	1	1	456.35
12	25	3	3	10	1.8	24	−1	−1	−1	−1	−1	271.60
12C	35	5	4.5	20	3.6	48	0	0	0	0	0	387.40
14C	35	5	4.5	20	3.6	48	0	0	0	0	0	419.30
15C	35	5	4.5	20	3.6	48	0	0	0	0	0	355.50

[a]Due to the copresence of two (hemi)cellulolytic activities in the *P. ostreatus* supernatant, when 10, 20, and 30 U/gds of cellulase are utilized in the different experimental runs, the xylanase concentrations in the enzymatic hydrolytic mixtures are 200, 400, and 600 U/gds, respectively.

Table 2: 3^3 experimental screening design: three levels, 3 factors, and 3 central points.

Run	T (°C)	Time (hours)	pH	Total sugars released (mg/gds)
1	35	48	2.5	289.90
2	35	48	3	264.40
3	35	48	3.5	353.30
4	35	72	2.5	184.60
5	35	72	3	180.60
6	35	72	3.5	315.80
7	35	96	2.5	187.50
8	35	96	3	233.80
9	35	96	3.5	335.90
10	45	48	2.5	196.90
11	45	48	3	209.10
12	45	48	3.5	233.90
12	45	72	2.5	150.30
14	45	72	3	207.40
15	45	72	3.5	291.70
16	45	96	2.5	292.80
17	45	96	3	347.40
18	45	96	3.5	480.10
19	55	48	2.5	69.40
20	55	48	3	185.0
21	55	48	3.5	235.60
22	55	72	2.5	156.70
23	55	72	3	155.50
24	55	72	3.5	156.60
25	55	96	2.5	203.18
26	55	96	3	262.0
27	55	96	3.5	281.0
28C	45	72	3	210.9
29C	45	72	3	211.0
30C	45	72	3	210.5

3. Results and Discussion

3.1. Analysis of Inducers of Cellulase and Xylanase Production in P. ostreatus. A preliminary screening of the potential inducers of cellulase and xylanase activities production in *P. ostreatus* was firstly performed in 24-well plates monitoring the time course of the enzymes secretion for 14 days in the presence of 1% (w/v) CMC, microcrystalline cellulose, cellobiose, sophorose, xylan, xylose, xylitol, wheat arabinoxylan, galactose, lactose, and 0.6 mM sophorose. Many researches have been focused on induction of cellulase and xylanase production by different monosaccharides, disaccharides, and carbohydrates used as sole carbon source [16]. Generally, the final concentration of the sugar tested as inducer corresponds to the 1% (w/v), demonstrating that this amount gives an inductive effect on both cellulases [17, 18] and xylanases [19, 20]. As regards the sophorose, although in most cases it was used as carbon source at final concentration of 1% (w/v) [21–23], its inductive effect on cellulase activity was also reported at lower concentration [24].

This analysis revealed the effect of microcrystalline cellulose as inducer of cellulase and xylanase activities production (Figures S1 A-B), whilst no induction was evidenced in the tested conditions by the other compounds, although in literature they have been reported as strong inducers of (hemi)cellulases production by fungi, such as *Trichoderma reesei* [25], *Clostridium acetobutylicum* [26], and *Aspergillus niger* [27].

Based on the data obtained from the analyses in multiwell, *P. ostreatus* cultures in 1 L Erlenmeyer flasks containing 500 mL of medium with 1% microcrystalline cellulose were performed (Figures S2 A-B). The cellulase and xylanase activities reached a maximum value of 3.19 and 51.32 U/mL, respectively, after 9 days of fermentation. In many works, microcrystalline cellulose was reported as an inducer of cellulase and xylanase activities production in fungal strains, such as *Cerrena unicolor* VKM F-3196 [28], *Trichoderma viride* [29], *Streptomyces* sp. [30], and *Alternaria brassicae* [31]. When *C. unicolor* VKM F-3196 was grown in a medium containing microcrystalline cellulose, a production of 6.5 U/mL xylanase and 12.7 U/mL cellulase, at the fifth day of fermentation, was observed by Belova et al. [28]. Similar amounts of cellulase were produced by *Trichoderma viride*, cultivated on microcrystalline cellulose, reaching the highest value of 10.19 U/mg after 3 days of growth [29]. During submerged fermentation in presence of microcrystalline cellulose, *Streptomyces* sp. EC22 produced a maximum of cellulase (0.8 U/mL) and xylanase (2.4 U/mL) activity after 72 and 60 hours, respectively [30]. Ortega [31] showed the induction of extracellular cellulolytic enzymes for the fungus *Alternaria brassicae* grown in the presence of microcrystalline cellulose with a maximum of 1.95 and 10.90 U/mL for endoglucanase and xylanase activities, respectively, very low values in comparison with those hereby reported for the strain *P. ostreatus*.

3.2. Partial Characterization of P. ostreatus Cellulase and Xylanase Activities to Define the Enzymatic Properties Useful for Their Application. The analysis of the culture supernatant obtained after 9 days of growth of *P. ostreatus* revealed only very low α-arabinofuranosidase, β-glucosidase, and β-xylosidase activities whose concentrations were of 0.0082, 0.056, 0.0076 U/mL, respectively. These three enzymatic activities are needed to obtain the biomass bioconversion into fermentable sugars.

Moreover, the optimal temperature and pH, thermoresistance, and pH-stability of cellulase and xylanase activities produced by *P. ostreatus* were also evaluated to identify the conditions to be tested in the statistical analysis of *A. donax* bioconversion.

In McIlvaine buffer, the optimal pH for the *P. ostreatus* cellulase activity was 4.0 (Figure 1(a)), similar to that reported for the three cellulases produced by *P. florida* (pH of 4.4) [32]. At least 60% of *P. ostreatus* cellulase activity was maintained in the range 3.0–7.0. Differently, the xylanase enzyme lost completely the activity at pH 3.0 and pH 4.0, showing an

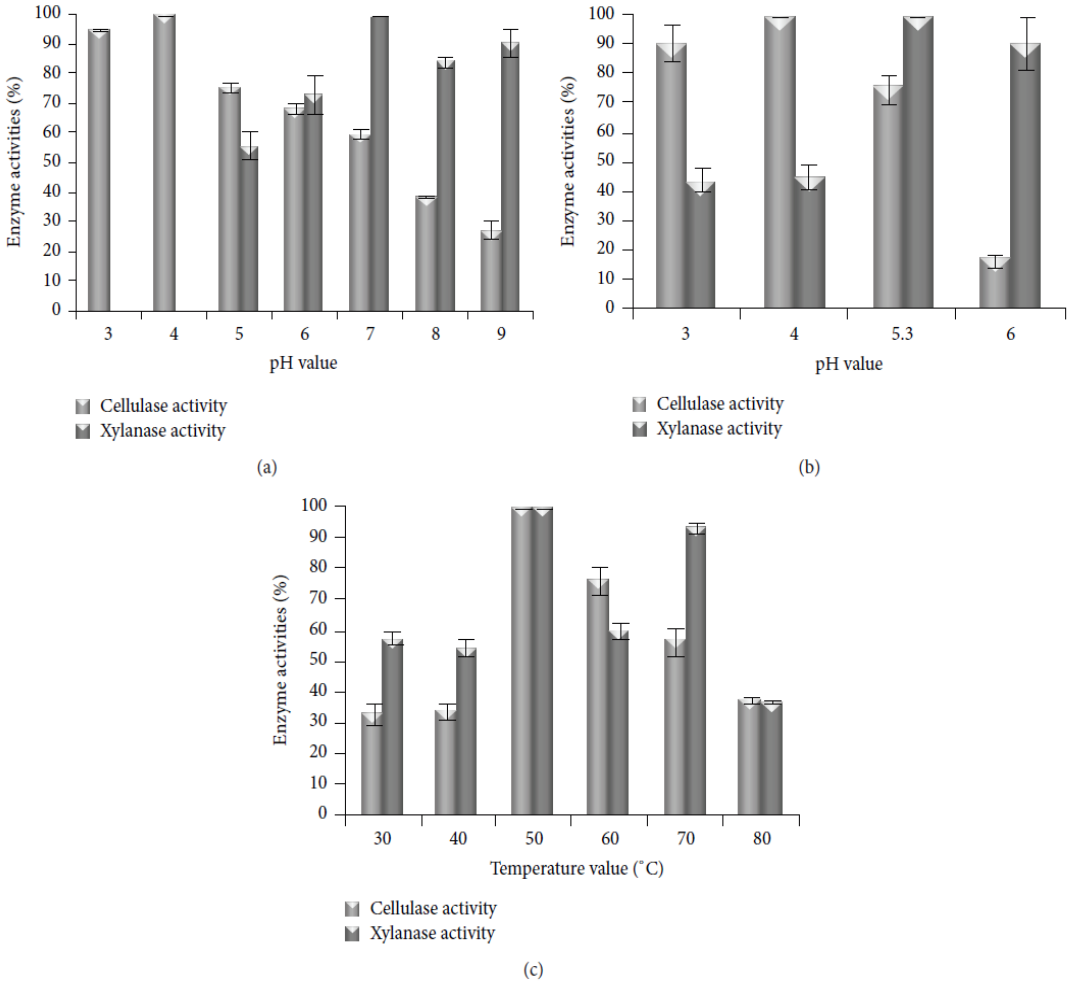

FIGURE 1: Effect of (a) pH in McIlvaine buffer, (b) pH in Na-citrate buffer, and (c) temperature on *Pleurotus ostreatus* cellulase and xylanase activities. The cellulase and xylanase activities were measured at pH ranging from 3.0 to 9.0 in McIlvaine buffer and from 3.0 to 6.0 in Na-citrate buffer and at the temperatures from 30 to 80°C.

optimum at 7.0 (Figure 1(a)), a value close to the optimum of 6.0 reported for a xylanase produced by *P. ostreatus* [33].

In the Na-citrate buffer, the cellulase activity analyzed in our work showed an optimum at same pH value of 4.0, while a different optimal pH of 5.3 was observed for the xylanase (Figure 1(b)).

Both the *P. ostreatus* cellulase and xylanase activities showed an optimal temperature of 50°C (Figure 1(c)), which represents the condition mostly used for the enzymatic hydrolysis of lignocellulosic biomasses [15, 34]. The results were comparable to the optimum temperature of 45°C shown by the three cellulases produced by *P. florida* [32], while it was distant from that of xylanase produced by *P. ostreatus* (between 25 and 40°C) [33].

The *P. ostreatus* cellulase and xylanase activities showed very high stability in a broad range of pH values (Figures 2(a) and 2(b)). It is worth noting that the xylanase showed a more elevated stability than the other xylanase produced by *P. ostreatus* that loses 15 and 22% of activity at pH 6.5 and pH 9.0, respectively, after only 3 hours [33].

Cellulase activity from *P. ostreatus* retained 50% of its value for at least 7 hours at 30°C and 40°C and 2 hours at 50°C and it immediately lost activity at temperature higher than 70°C (Figure 3(a)); other cellulases from *Pleurotus* sp., β-glucosidase, endoglucanase, and exoglucanase, showed a half-life of 15 minutes at 72, 66, and 58°C, respectively [32]. Xylanase activity from *P. ostreatus* retained 50% of its activity for at least 2 days at 30°C and 7 hours at 40°C (Figure 3(a)); like the other xylanase from *P. ostreatus* [33], it lost the activity at temperatures higher than 50°C.

3.3. Screening of Parameters Affecting the Enzymatic Hydrolysis by PB Design. Statistical analysis of *Arundo donax* bioconversion by cellulases and xylanases produced by *Pleurotus*

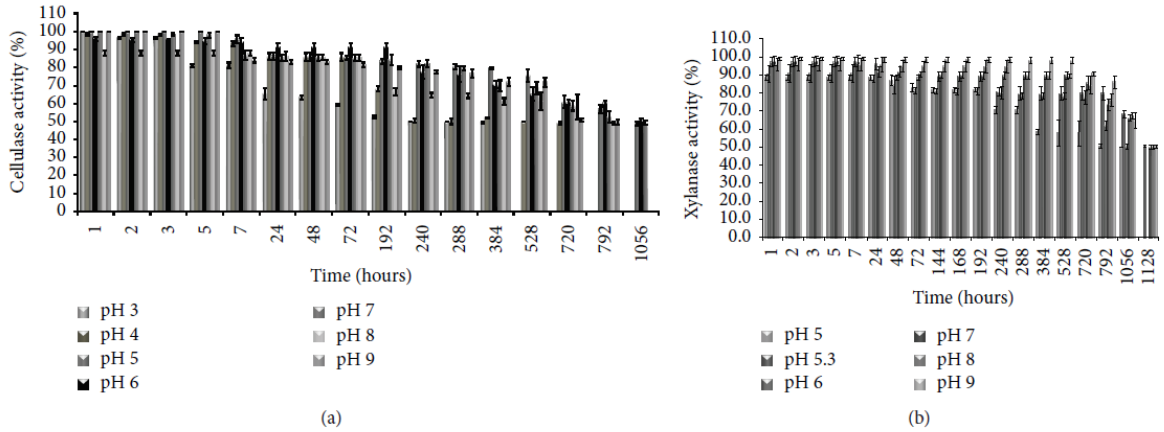

FIGURE 2: pH resistance of (a) cellulase and (b) xylanase activities of *Pleurotus ostreatus*. The pH resistance of the cellulase and xylanase activities was analyzed by diluting the supernatant in McIlvaine buffer, with pH ranging from 3.0 to 9.0, and incubating at 25°C. The percentage values reported in the graphs are referred to the initial enzymatic activities of 100%.

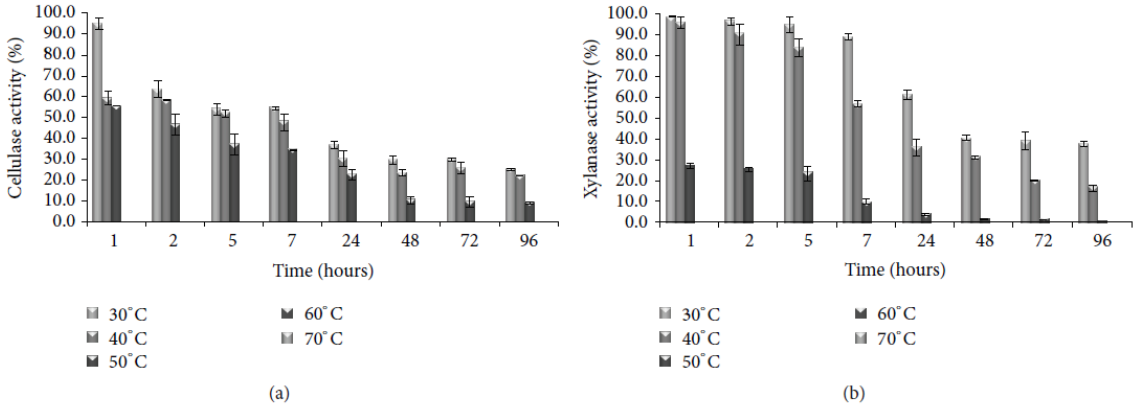

FIGURE 3: Thermoresistance of (a) cellulase and (b) xylanase activities of the strain *Pleurotus ostreatus*. The thermoresistance of the xylanase and cellulase activities was investigated by incubating the fungal culture supernatant in 50 mM Na citrate pH 5.3, at 30, 40, 50, 60, 70, and 80°C. The percentage values reported in the graphs are referred to the initial enzymatic activities of 100%.

TABLE 3: Macromolecular composition of untreated and pretreated *Arundo donax*.

	Carbohydrate composition (% total dry weight)	
	Untreated	Pretreated
Glucan	26.3 ± 1.6	38.2 ± 1.2
Xylan	24.1 ± 1.2	5.7 ± 0.9
Klason lignin	9.8 ± 0.4	36.1 ± 0.6

ostreatus after 9 days of growth in the presence of microcrystalline cellulose was performed. Carbohydrate compositions of the untreated and pretreated [35] giant reed (*Arundo donax*) are reported in Table 3.

The effect of the six parameters, temperature (°C), biomass (%, w/v), pH, cellulase from *P. ostreatus* (U/gds), commercial cellulase from *Trichoderma reesei* ATCC26921 (U/gds), and incubation time (hours), on the sugars released during the enzymatic hydrolysis by *P. ostreatus* extracellular cellulase and xylanase was analyzed through the PB screening design.

Since in the enzymatic cocktail produced by *Pleurotus ostreatus* after 9 days of growth the cellulase activities were lower than the xylanase one, the only cellulase activity level was chosen as parameter in the statistical analysis experiments.

Moreover, the addition of the commercial enzymatic mix C (145 U/gds of cellobiase from *A. niger* and 8 U/gds of thermostable β-xylosidase) in the hydrolysis reaction was necessary to obtain the complete carbohydrates hydrolysis into

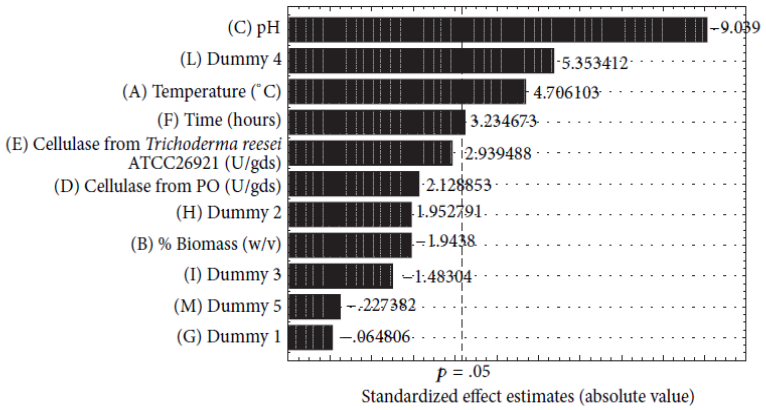

FIGURE 4: Pareto Chart of the Plackett-Burman design of sugars released during the enzymatic hydrolysis of *Arundo donax* (R^2 = 0.98 and $R^2_{adj.}$ = 0.92), showing the significant factors at 95% of confidence level ($p < 0.05$). Significant factors were pH (p = 0.002859), temperature (p = 0.018157), and time (p = 0.048045) with an effect of −155.83, +104.69, and +71.96, respectively.

the respective monomers, since the β-glucosidase and β-xylosidase activities were not detected in the supernatant of the strain *P. ostreatus*.

In Table S1 the lowest (−1) and the highest (+1) values tested for each factor were reported. The results of the analysis were reported in Table 1, ANOVA data were reported in Table S3, and the Pareto Chart, showing the standardized effects of the analyzed factors, was presented in Figure 4.

As reported in the ANOVA Table (Table S3), the most significant influence on the released sugars during the enzymatic hydrolysis of *A. donax* was exercised by the temperature (°C), pH, and time. In Figure 4, the Pareto Chart (R^2 = 0.98 and $R^2_{adj.}$ = 0.92) shows that the three significant factors at 95% of confidence level ($p < 0.05$) are the pH representing the most important factor for the released sugars (p = 0.002859) and exercising a strong negative effect of −155.83, followed by the temperature (p = 0.018157) and the time (p = 0.048045) with a positive effect of +104.69 and +71.96, respectively.

Within the tested experimental values, the other three variables, dosage of cellulase from *T. reesei* ATCC 26921, dosage of cellulase from *P. ostreatus*, and % (w/v) of biomass, did not show significant effect at 95% of confidence level on the hydrolysis of saccharification of *A. donax*. The dosage of cellulase from *Trichoderma reesei* ATCC 26921 and that from *P. ostreatus* affected the saccharification of *A. donax* at 94 and 88% confidence levels, respectively.

Moreover, the PB analysis showed a strong influence of the dummy 4, one of the unassigned considered variables. As explained by Stowe and Mayer [36] (1966), this may be due to an experimental error or a possible interaction between two factors not revealed by the PB analysis but by the 3^3 full factorial analysis.

3.4. Analysis of Combined Effect of pH, Temperature, and Time on the Bioconversion of Arundo donax through the 3^3 Factorial Experimental Design and Validation of Generated Model. Since the PB analysis showed that the pH, temperature (°C), and time (hours) were the most significant factors on the sugars released during the enzymatic hydrolysis of *A. donax*, a 3^3 full factorial experimental design with 30 runs was performed to analyze the combined effect of them.

As regards the other factors, % (w/v) of biomass, U/gds of cellulase from *P. ostreatus*, and U/gds of commercial cellulase from *T. reesei* ATCC26921, since they had no effect on the released sugars, the experiments were performed using their level 0 tested in the PB analysis, in order to reduce the utilization of both commercial cellulase and enzymes from *P. ostreatus*, with economical advantage. Also, for the % (w/v) of biomass, it was chosen to adopt the level 0 (5% w/v) since previous experiments had shown better results with this biomass percentage [37].

In Table S2, the lowest (−1) and the highest (+1) values tested for each factor in the 3^3 full factorial experimental design were reported. The results of the analysis were reported in Table 2, ANOVA data were reported in Table S4, and the Pareto Chart, showing the standardized effects of the analyzed factors, was presented in Figure 5.

The best result of 480.10 mg of sugars/gds was given by the following combination of factors (run number 18): 45°C, pH 3.5, and 96 hours of incubation (Figure 5).

The ANOVA Table (Table S4) showed that all the tested factors (temperature, pH, and time) exercised a strong influence on the sugars released during the enzymatic hydrolysis of *A. donax*. The 95% confidence interval and a coefficient of determination R^2 of 0.93, ($R^2_{adj.}$ of 0.80) indicated that the model was statistically significant. As shown in the Pareto Chart (Figure 5), the pH was the most significant factor for the released sugars (p = 0.000183), in agreement with the results obtained by PB analysis, and it exercised a strong negative effect of −105.85; it is followed by the temperature (p = 0.000921) and the time (p = 0.000537) with a negative effect of −71.20 and a positive effect of +65.13, respectively.

A relevant positive impact on the bioconversion process was exercised by the interaction between temperature and

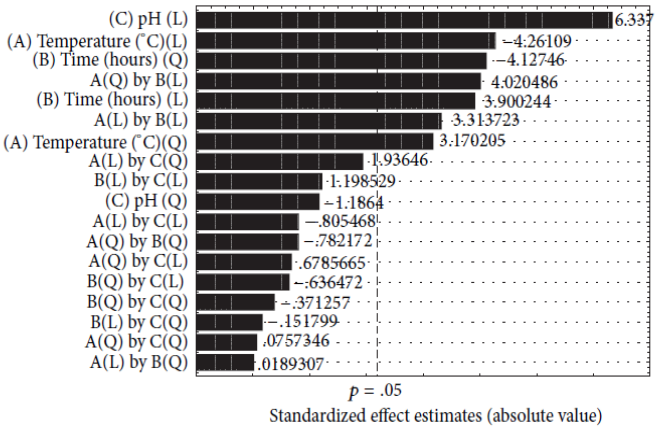

FIGURE 5: Pareto Chart of the 3^3 full factorial design of sugars released during the enzymatic hydrolysis of *Arundo donax* ($R^2 = 0.93$; $R^2_{adj.} = 0.80$), showing the significant factors at 95% of confidence level ($p < 0.05$). Significant factors were pH ($p = 0.000183$), temperature ($p = 0.000921$), and time ($p = 0.000537$) with an effect of −105.85, −71.20, and +65.13, respectively. Strong positive impact on the bioconversion process was exercised also by the interaction between the temperature and the time.

time. This strong interaction could explain the influence of the dummy 4 revealed by the PB analysis, excluding the hypothesis of an experimental error [36].

The 3D response surface was obtained by plotting the response values (mg glucose/g dry substrate) on the Z axis against (1) the variables temperature and time, keeping the pH constant at its level 0 (Figure 6(a)), (2) the variables pH and time, keeping the temperature constant at its level 0 (Figure 6(b)), and (3) the variables temperature and pH, keeping the time constant at its level 0 (Figure 6(c)). The Surface Response is demonstrated by

$$\begin{aligned} Y = {} & 206.25 - 12.49 * A - 30.81 * A^2 + 78.27 * B \\ & + 79.39 * B^2 + 53.44 * C + 22.14 * C^2 + 33.90 \\ & * AB - 8.25 * AC - 0.33 * AB^2 - 34.32 * AC^2 \\ & - 71.23 * A^2B - 12.04 * A^2C - 22.22 * A^2B^2 \\ & + 2.13 * A^2C^2 + 12.24 * BC + 2.67 * BC^2 \\ & + 11.26 * B^2C - 10.56 * B^2C^2, \end{aligned} \quad (2)$$

where Y indicates the released sugars concentration (mg/g dry substrate), A is the temperature (°C), B is the time (hours), and C is the pH.

The saccharification ratio increased with increase in time of incubation, keeping constant the pH at 3.5. Increase of temperature in the range from 35 to 45°C resulted in an improvement of the sugars yield. This trend, due to the positive interaction between temperature and time, gave more pronounced effect at longer incubation times with the maximum sugar recovery at 96 h. At this time a further temperature increase generated a reduction of saccharification yield (Figure 6).

The experimental results (average) did not differ significantly with the theoretical values obtained by the generated model (Table S5). Based on the determination coefficient ($R^2 = 0.93$ and $R^2_{adj.} = 0.80$), more than 80% of the results are represented by the model. The plotting between the experimental total reducing sugars and the predicted total reducing sugars was reported in Figure S3.

The conditions under which the maximum value of the sugars released was achieved were also verified by carrying out the experiments corresponding of run 18, whose results were in close agreement with the model prediction (455 mg/gds).

In general, the sugars released by the bioconversion of the pretreated *A. donax* reported in this study were not so far from those reported until now in literature, also in comparison with the results obtained using commercial enzymes. Several sources of enzymes were reported in the bioconversion of lignocellulosic biomasses and the different sugars yield obtained, which are summarized in Table 4, were influenced by the type of pretreated lignocellulosic materials, the enzymatic cocktail, and the operative conditions adopted in the process.

It is worth noting that the bioconversion of the pretreated *A. donax* reported in this study released similar amounts of glucose (229.30 mg/gds) and xylose (250.80 mg/gds) to those previously obtained by using commercial enzymes cocktail, containing 60 FPU/gds of cellulases and 64 pNPGU/gds of β-glucosidases, in the bioconversion of the same biomass, which released 264.0 mg glucose/gds and 217.0 mg xylose/gds [38] (Table 4). Rana et al. [39] showed that the enzymatic bioconversion of biological pretreated *Parthenium sp.* with the Accellerase 1500 (Novozymes) allowed obtaining 485.64 mg of total sugars per gram of dry substrate (Table 4), comparable to that (480.10 mg/gds) obtained in this study.

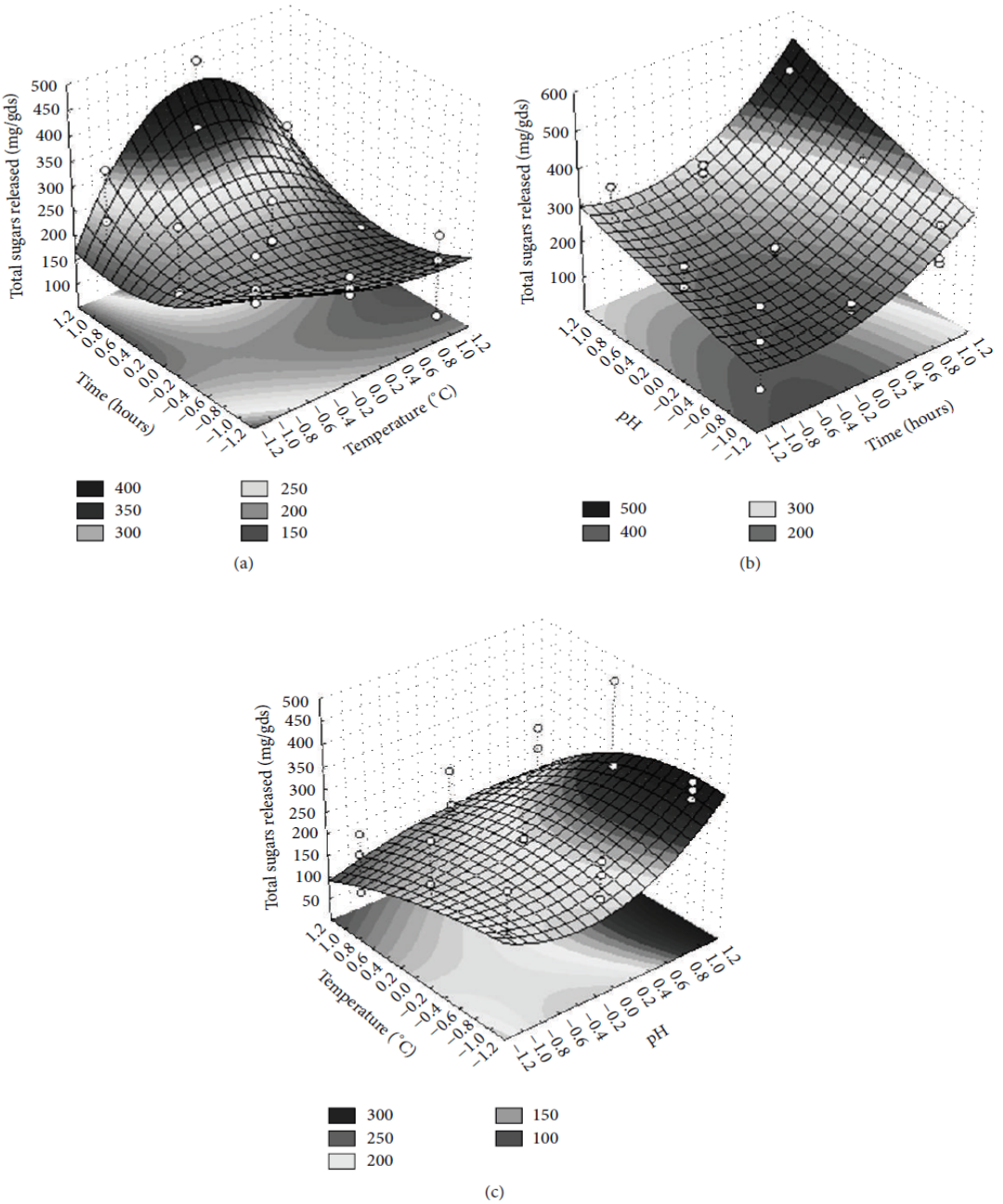

FIGURE 6: Surface Response showing the optimum region of each variable (central point) for the highest sugars released (mg/gds) during the hydrolysis of *Arundo donax*.

Table 4: Comparison of enzymatic hydrolysis of different pretreated lignocellulosic biomasses by different enzyme sources and the obtained sugars released (mg/gds).

Biomass	Pretreatment	Glucose release (mg/gds)	Xylose release (mg/gds)	Enzyme used	References
Narrow-leaf cattail	Alkali pretreatment	552.9	74.0	13.50 FPU/g of *Trichoderma reesei* ATCC 26921 and 16.50 U/g β-glucosidase enzyme by *Almonds Lyophyl*	[46]
Corn cob	Alkali pretreatment	438.47		717.0 U/g of xylanase, 77 U/gds of CMCase, and 26.12 U/gds of FPase by *Aspergillus candidus*	[40]
Wheat straw Rice straw Corncobs	Alkali pretreatment	193.86 178.93 171.06		2400 U/g of xylanase from *Aspergillus foetidus* MTCC 4898	[41]
Apple pomace	Acid pretreatment and polyphenol degradation	266.0	—	43.0 U/g of Celluclast 1.5 L, 183.0 U/g of Pectinex 3XL, and 41.0 U/g of Novozyme 188 (Novozymes)	[44]
Cistus ladanifer *Cytisus striatus*	Acid pretreatment	313.0 448.0	—	60.0 FPU/g of Cellulase complex NS50013 and * U/g β-glucosidase NS50010 (Novozymes)	[45]
Oil palm empty fruit bunches	Alkali pretreatment	534.53		10% (v/v) of crude cellulase enzyme by *Trichoderma reesei* RUT C-30	[47]
Populus nigra	Alkali pretreatment	667.0		25 FPU/g of cellulase by *Pholiota adiposa* SKU0714	[48]
Parthenium sp.	Alkali pretreatment	513.0		7 FPU/g of Accellerase 1500 (Genencor)	[49]
Sweet sorghum straw	Acid pretreatment	440.0	—	25 FPU/g of cellulase by *Coriolus versicolor* TD17	[43]
Gracilaria sp.	Acid pretreatment	592.0		0.01 g/g of commercial cellulase	[50]
Paddy straw or *Parthenium* sp.	Biological pretreatement by *Myrotechium roridum* LG7	509.65		* U/g of Accellerase 1500 (Novozymes)	[49]
Arundo donax	Acid pretreatment	264.0	217.0	60 FPU/g cellulose of cellulases and 64 pNPGU/g cellulose β-glucosidases	[38]
Parthenium sp.	Biological pretreatement by *Trametes hirsuta*	485.64		* U/g of Accellerase 1500 (Novozymes)	[39]
Corn stover	Acid pretreatment	545.0		* U/g of Accellerase 1500 and * U/g of Accellerase XY (Genencor)	[51]
Populus balsamifera	Alkali pretreatments	293.0		100 FPU/g of cellulase by *Agaricus arvensis*	[42]
Arundo donax	Steam explosion	229.3 480.10	250.8	20 U/gds and 400 U/gds of cellulase and xylanase, respectively, from *Pleurotus ostreatus*, supplemented with the commercial enzymatic mix C	This study

*In the paper the enzymatic units were not reported.

In some cases, the amount of sugars obtained using crude enzymes from fungi in lignocellulose bioconversion, that is, from *Aspergillus candidus* in saccharification of aqueous ammonia treated corn cob or from *Aspergillus foetidus* MTCC 4898 in saccharification of NaOH pretreated agricultural residues like wheat straw, rice straw, and corncobs [40, 41], was lower than that obtained in our study (Table 4).

Many works concerning the statistical optimization of pretreated lignocellulosic materials bioconversion, applying either commercial enzymes or enzymes from different microorganisms, were so far reported in literature. Lower sugars yield (293 mg/g substrate) than those reported in this work were obtained from the saccharification of *Populus balsamifera*, under optimized conditions, 65 FPU of cellulases from *Agaricus arvensis*/g substrate, 10% of the substrate, and a temperature of 40°C [42] (Table 4); while higher sugars yield, comparable to those obtained by using *P. ostreatus* enzymes, was reported by Phuengjayaem et al. [43].

It is noteworthy that the sugars' yield hereby reported with *P. ostreatus* enzymes was significant also when compared with that reached by process optimization with commercial enzymes. In fact, maximum reducing sugar yield of 266.0 mg/g was found, under optimized conditions, for the enzymatic hydrolysis of apple pomace by a commercial cocktail (43.0 U/g of Celluclast 1.5 L, 183.0 U/g of Pectinex 3XL, and 41.0 U/g of Novozyme 188), as reported by Parmar and Rupasinghe [44] (Table 4). Ferreira et al. [45], through response surface methodology, obtained a maximum reducing sugar yield of 313.0 mg/g for *Cistus ladanifer* and 418.0 mg/g for *Cytisus striatus* by using 60.0 FPU/g of cellulase complex NS50013 and β-glucosidase NS50010 (Table 4).

Moreover, at the best assessed conditions, the enzymatic cocktail produced by *P. ostreatus* allowed obtaining, at the same time, both glucose and xylose at similar yields. Differently, Ruangmee and Sangwichien [46] showed that at the optimal conditions for glucose yield (552.9 mg/g substrate), the xylose yield (74.0 mg/g substrate) was much lower.

Furthermore, it is worthy to note that an extensive literature search reveals that (hemi)cellulolytic enzymes produced by *P. ostreatus* represent a good candidate as biocatalyst for the enzymatic saccharification of *A. donax*, since they give a sugar yield that was only in few cases a little bit lower [47, 49] or comparable [40, 43] whilst, in some other cases, it results higher than that obtained by using enzymes from different sources [47–49], including the commercial ones [50–52].

4. Conclusions

It was demonstrated that microcrystalline cellulose is able to induce the *Pleurotus ostreatus* production of a mixture of cellulases and xylanases which were shown to be able to hydrolyze the pretreated *Arundo donax*. A statistical analysis of bioconversion based on this mixture led to the best results of 480.10 mg of sugars/gds at 45°C, pH 3.5, and 96 hours of incubation. In these conditions, a significant sugar yield was obtained also in comparison with results previously reported in literature. Interestingly, both glucose and xylose at similar yields were achieved at the same time.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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References

[1] R. Liguori, A. Amore, and V. Faraco, "Waste valorization by biotechnological conversion into added value products," *Applied Microbiology and Biotechnology*, vol. 97, no. 14, pp. 6129–6147, 2013.

[2] S. Prasad, A. Singh, and H. C. Joshi, "Ethanol as an alternative fuel from agricultural, industrial and urban residues," *Resources, Conservation and Recycling*, vol. 50, no. 1, pp. 1–39, 2007.

[3] S. M. Duncan and J. S. Schilling, "Carbohydrate-hydrolyzing enzyme ratios during fungal degradation of woody and non-woody lignocellulose substrates," *Enzyme and Microbial Technology*, vol. 47, no. 7, pp. 363–371, 2010.

[4] A. Amore, B. Parameswaran, R. Kumar et al., "Application of a new xylanase activity from *Bacillus amyloliquefaciens* XR44A in Brewer's spent grain saccharification," *Journal of Chemical Technology and Biotechnology*, vol. 90, no. 3, pp. 573–581, 2015.

[5] A. Amore, O. Pepe, V. Ventorino, L. Birolo, C. Giangrande, and V. Faraco, "Cloning and recombinant expression of a cellulase from the cellulolytic strain *Streptomyces* sp. G12 isolated from compost," *Microbial Cell Factories*, vol. 11, article 164, 2012.

[6] A. Amore, O. Pepe, V. Ventorino, L. Birolo, C. Giangrande, and V. Faraco, "Industrial waste based compost as a source of novel cellulolytic strains and enzymes," *FEMS Microbiology Letters*, vol. 339, no. 2, pp. 93–101, 2013.

[7] S. Giacobbe, O. Pepe, V. Ventorino, L. Birolo, R. Vinciguerra, and V. Faraco, "Identification and characterisation of a pectinolytic enzyme from *Paenibacillus xylanolyticus*," *BioResources*, vol. 9, no. 3, pp. 4873–4887, 2014.

[8] M. Kostylev and D. Wilson, "Synergistic interactions in cellulose hydrolysis," *Biofuels*, vol. 3, no. 1, pp. 61–70, 2012.

[9] A. Amore, S. Giacobbe, R. Liguori, and V. Faraco, "The second generation ethanol production," in *Rendiconti della Accademia Nazionale delle Scienze detta dei XL. Memorie di Scienze Fisiche e Naturali*, pp. 113–136, 2014.

[10] M. Fagnano, N. Diodato, I. Alberico, and N. Fiorentino, "An overview of soil erosion modeling compatible with RUSLE approach," *Rendiconto delle Scienze Fisiche Accademia dei Lincei*, vol. 23, pp. 69–80, 2012.

[11] M. Fagnano, A. Impagliazzo, M. Mori, and N. Fiorentino, "Agronomic and environmental impacts of giant reed (*Arundo donax* L.): results from a long-term field experiment in hilly areas subject to soil erosion," *Bioenergy Research*, vol. 8, no. 1, pp. 415–422, 2015.

[12] A. Forte, A. Zucaro, M. Fagnano, S. Bastianoni, R. Basosi, and A. Fierro, "LCA of *Arundo donax* L. lignocellulosic feedstock production under Mediterranean conditions," *Biomass and Bioenergy*, vol. 73, pp. 32–47, 2015.

[13] M. J. Bailey, P. Biely, and K. Poutanen, "Interlaboratory testing of methods for assay of xylanase activity," *Journal of Biotechnology*, vol. 23, no. 3, pp. 257–270, 1992.

[14] T. K. Ghose, "Measurement of cellulase activities," *Pure and Applied Chemistry*, vol. 59, pp. 257–268, 1987.

[15] L. Marcolongo, E. Ionata, F. La Cara et al., "The effect of *Pleurotus ostreatus* arabinofuranosidase and its evolved variant in lignocellulosic biomasses conversion," *Fungal Genetics and Biology*, vol. 72, pp. 162–167, 2014.

[16] A. H. Bahkali, "In vitro production of pectolytic and cellulolytic enzymes by *Colletotrichum lindemuthianum* isolated from soybean grown in Saudi Arabia," *World Journal of Microbiology & Biotechnology*, vol. 8, no. 1, pp. 55–59, 1992.

[17] E. A. Ximenes, C. R. Felix, and C. J. Ulhoa, "Production of cellulases by *Aspergillus fumigatus* and characterization of one β-glucosidase," *Current Microbiology*, vol. 32, no. 3, pp. 119–123, 1996.

[18] M. I. Rajoka and K. A. Malik, "Enhanced production of cellulases by *Cellulomonas* strains grown on different cellulosic residues," *Folia Microbiologica*, vol. 42, no. 1, pp. 59–64, 1997.

[19] O. Pérez-Avalos, T. Ponce-Noyola, I. Magaña-Plaza, and M. de la Torre, "Induction of xylanase and β-xylosidase in *Cellulomonas glavigena* growing on different carbon sources," *Applied Microbiology and Biotechnology*, vol. 46, no. 4, pp. 405–409, 1996.

[20] C. Joshi and S. K. Khare, "Induction of xylanase in thermophilic fungi *Scytalidium thermophilum* and *Sporotrichum thermophile*," *Brazilian Archives of Biology and Technology*, vol. 55, no. 1, pp. 21–27, 2012.

[21] T. Nisizawa, H. Suzuki, M. Nakayama, and K. Nisizawa, "Inductive formation of cellulase by sophorose in *Trichoderma viride*," *Journal of Biochemistry*, vol. 70, no. 3, pp. 375–385, 1971.

[22] M. Ilmén, A. Saloheimo, M.-L. Onnela, and M. E. Penttilä, "Regulation of cellulase gene expression in the filamentous fungus *Trichoderma reesei*," *Applied and Environmental Microbiology*, vol. 63, no. 4, pp. 1298–1306, 1997.

[23] P. K. Foreman, D. Brown, L. Dankmeyer et al., "Transcriptional regulation of biomass-degrading enzymes in the filamentous fungus *Trichoderma reesei*," *Journal of Biological Chemistry*, vol. 278, no. 34, pp. 31988–31997, 2003.

[24] D. Sternberg and G. R. Mandels, "Induction of cellulolytic enzymes in *Trichoderma reesei* by sophorose," *Journal of Bacteriology*, vol. 139, no. 3, pp. 761–769, 1979.

[25] T. Portnoy, A. Margeot, V. Seidl-Seiboth et al., "Differential regulation of the cellulase transcription factors XYR1, ACE2, and ACE1 in *Trichoderma reesei* strains producing high and low levels of cellulase," *Eukaryotic Cell*, vol. 10, no. 2, pp. 262–271, 2011.

[26] M. D. Servinsky, J. T. Kiel, N. F. Dupuy, and C. J. Sund, "Transcriptional analysis of differential carbohydrate utilization by *Clostridium acetobutylicum*," *Microbiology*, vol. 156, no. 11, pp. 3478–3491, 2010.

[27] A. R. Stricker, R. L. Mach, and L. H. de Graaff, "Regulation of transcription of cellulases- and hemicellulases-encoding genes in *Aspergillus niger* and *Hypocrea jecorina* (*Trichoderma reesei*)," *Applied Microbiology and Biotechnology*, vol. 78, no. 2, pp. 211–220, 2008.

[28] O. V. Belova, A. V. Lisov, N. G. Vinokurova et al., "Xylanase and cellulase of fungus *Cerrena unicolor* VKM F-3196: production, properties, and applications for the saccharification of plant material," *Prikladnaia Biokhimiia i Mikrobiologiia*, vol. 50, no. 2, pp. 171–176, 2014.

[29] Y. Feng, H.-Q. Liu, F. Xu, and J.-X. Jiang, "Enzymatic degradation of steam-pretreated *Lespedeza* stalk (*Lespedeza cryptobotrya*) by cellulosic-substrate induced cellulases," *Bioprocess and Biosystems Engineering*, vol. 34, no. 3, pp. 357–365, 2011.

[30] B. C. Okeke and A. Paterson, "Simultaneous production and induction of cellulolytic and xylanolytic enzymes in a *Streptomyces* sp," *World Journal of Microbiology & Biotechnology*, vol. 8, no. 5, pp. 483–487, 1992.

[31] J. Ortega, "Cellulases of the phytopathogenic fungus *Alternaria brassicae*," *Texas Journal of Science*, vol. 44, no. 3, pp. 313–316, 1992.

[32] M. Goyal and G. Soni, "Production and characterization of cellulolytic enzymes by *Pleurotus florida*," *African Journal of Microbiology Research*, vol. 5, no. 10, pp. 1131–1136, 2011.

[33] C. Qinnghe, Y. Xiaoyu, N. Tiangui, J. Cheng, and M. Qiugang, "The screening of culture condition and properties of xylanase by white-rot fungus *Pleurotus ostreatus*," *Process Biochemistry*, vol. 39, no. 11, pp. 1561–1566, 2004.

[34] G. Banerjee, S. Car, J. S. Scott-Craig, M. S. Borrusch, M. Bongers, and J. D. Walton, "Synthetic multi-component enzyme mixtures for deconstruction of lignocellulosic biomass," *Bioresource Technology*, vol. 101, no. 23, pp. 9097–9105, 2010.

[35] R. Di Pasqua, V. Ventorino, A. Aliberti et al., "Influence of different lignocellulose sources on endo-1,4-β-glucanase gene expression and enzymatic activity of *Bacillus amyloliquefaciens*," *BioResources*, vol. 9, no. 1, pp. 1303–1310, 2013.

[36] R. A. Stowe and R. P. Mayer, "Mayer efficient screening of process variables," *Industrial & Engineering Chemistry*, vol. 58, pp. 36–40, 1966.

[37] L. Maurelli, E. Ionata, F. La Cara, and A. Morana, "Chestnut shell as unexploited source of fermentable sugars: effect of different pretreatment methods on enzymatic saccharification," *Applied Biochemistry and Biotechnology*, vol. 170, no. 5, pp. 1104–1118, 2013.

[38] A. A. Shatalov and H. Pereira, "Xylose production from giant reed (*Arundo donax* L.): modeling and optimization of dilute acid hydrolysis," *Carbohydrate Polymers*, vol. 87, no. 1, pp. 210–217, 2012.

[39] S. Rana, R. Tiwari, A. Arora et al., "Prospecting *Parthenium* sp. pretreated with *Trametes hirsuta*, as a potential bioethanol feedstock," *Biocatalysis and Agricultural Biotechnology*, vol. 2, no. 2, pp. 152–158, 2013.

[40] D. Garai and V. Kumar, "A Box-Behnken design approach for the production of xylanase by *Aspergillus candidus* under solid state fermentation and its application in saccharification of agro residues and *Parthenium hysterophorus L*," *Industrial Crops and Products*, vol. 44, pp. 352–363, 2013.

[41] D. Chapla, J. Divecha, D. Madamwar, and A. Shah, "Utilization of agro-industrial waste for xylanase production by *Aspergillus foetidus* MTCC 4898 under solid state fermentation and its application in saccharification," *Biochemical Engineering Journal*, vol. 49, no. 3, pp. 361–369, 2010.

[42] M. Jeya, N.-P. Nguyen, H.-J. Moon, S.-H. Kim, and J.-K. Lee, "Conversion of woody biomass into fermentable sugars by cellulase from *Agaricus arvensis*," *Bioresource Technology*, vol. 101, no. 22, pp. 8742–8749, 2010.

[43] S. Phuengjayaem, A. Poonsrisawat, A. Petsom, and S. Teeradakorn, "Optimization of saccharification conditions of acid-pretreated sweet sorghum straw using response surface methodology," *The Journal of Agricultural Science*, vol. 6, no. 9, pp. 120–133, 2014.

[44] I. Parmar and H. P. V. Rupasinghe, "Bio-conversion of apple pomace into ethanol and acetic acid: enzymatic hydrolysis and fermentation," *Bioresource Technology*, vol. 130, pp. 613–620, 2013.

[45] S. Ferreira, A. P. Duarte, M. H. L. Ribeiro, J. A. Queiroz, and F. C. Domingues, "Response surface optimization of enzymatic hydrolysis of *Cistus ladanifer* and *Cytisus striatus* for bioethanol production," *Biochemical Engineering Journal*, vol. 45, no. 3, pp. 192–200, 2009.

[46] A. Ruangmee and C. Sangwichien, "Response surface optimization of enzymatic hydrolysis of narrow-leaf cattail for bioethanol production," *Energy Conversion and Management*, vol. 73, pp. 381–388, 2013.

[47] S. S. Rashid and M. Z. Alam, "Statistical optimization of the enzymatic saccharification of the oil palm empty fruit bunches," *World Academy of Science, Engineering and Technology*, vol. 7, pp. 253–258, 2013.

[48] S. S. Jagtap, S. S. Dhiman, M. Jeya, Y. C. Kang, J.-H. Choi, and J.-K. Lee, "Saccharification of poplar biomass by using lignocellulases from *Pholiota adiposa*," *Bioresource Technology*, vol. 120, pp. 264–272, 2012.

[49] K. Pandiyan, R. Tiwari, S. Singh et al., "Optimization of enzymatic saccharification of alkali pretreated *Parthenium* sp. using response surface methodology," *Enzyme Research*, vol. 2014, Article ID 764898, 8 pages, 2014.

[50] F.-C. Wu, J.-Y. Wu, Y.-J. Liao, M.-Y. Wang, and I.-L. Shih, "Sequential acid and enzymatic hydrolysis in situ and bioethanol production from *Gracilaria biomass*," *Bioresource Technology*, vol. 156, pp. 123–131, 2014.

[51] Z. Ruan, M. Zanotti, Y. Zhong, W. Liao, C. Ducey, and Y. Liu, "Co-hydrolysis of lignocellulosic biomass for microbial lipid accumulation," *Biotechnology and Bioengineering*, vol. 110, no. 4, pp. 1039–1049, 2013.

[52] R. Tiwari, S. Rana, S. Singh et al., "Biological delignification of paddy straw and *Parthenium* sp. using a novel micromycete *Myrothecium roridum* LG7 for enhanced saccharification," *Bioresource Technology*, vol. 135, pp. 7–11, 2013.

Chapter III

Lignocellulosic waste conversion for lactic acid production

3.1 Introduction

Lactic acid is an organic acid recognized as a GRAS (Generally Recognized As Safe) compound by the US FDA (Food and Drug Administration) and it is widely applied in food, cosmetic, pharmaceutical and chemical industries [18]. The large amount of lactic acid produced, up to 70 %, is used in the food industry for cheese and yogurt production [19]. In the cosmetic industry, lactic acid is used as a component of hygiene and esthetic products, due to its verified antimicrobial and rejuvenating effects on the skin [20]. Moreover, in the pharmaceutical and chemical industries, lactic acid is used as a precursor of large and small polymers, like polylactic acid and acrylic polymers, which are biocompatible and biodegradable and thus, utilized for the packaging, prosthetic devices, sutures and internal drug dosing [21]. The worldwide demand of lactic acid in 2007 was calculated to be 130,000-150,000 metric tons per year, with commercial prices ranging between 1.38 US$ kg^{-1} and 1.54 US$ kg^{-1}, depending on the purity grade [22]. It was estimated that the lactic acid request should increase significantly over the coming years; thus, many efforts are focusing on the development of efficient and cost-effective processes to increase the lactic acid recovery yield. Particularly, low-cost lignocellulosic wastes are exploited as substrate for a competitive production of lactic acid through microbial fermentations [23,24,25].

Among the lignocellulosic wastes, Brewers' Spent Grain (BSG) is one of the most valuable low-cost feedstock for lactic acid production. BSG represents the major by-product of the brewing industry. It accounts for about 85 % of the total residues generated after the mashing and lautering processes [128] and it is available in large quantities throughout the year. The chemical composition of BSG varies according to several factors such as the barley variety, the harvest time, and the malting and mashing conditions.

This chapter deals with the description of studies concerning the conversion of BSG into lactic acid through microbial fermentation. These activities were carried out in the laboratory of Dr. Carlos Ricardo Soccol at the Department of "Engenharia de Bioprocessos e Biotecnologia" (DEBB) (Universidade Federal do Paraná, Brazil), in the frame of the project BIOASSORT.

Particularly, the paper II **"Selection of the strain *Lactobacillus acidophilus* ATCC 43121 and its application to Brewers' Spent Grain conversion into lactic acid"** describes the ability of the strain *Lactobacillus acidophilus* ATCC 43121, selected among six lactobacilli belonging to the DEBB collection, to produce lactic acid from BSG hydrolysates derived from two different geographical areas. In detail, the hydrolysates obtained by enzymatic hydrolysis of BSGs after two types of pretreatments, aqueous ammonia soaking and acid-alkaline pretreatment, were evaluated as substrate for the growth of the *Lactobacillus acidophilus* ATCC 43121 strain and the lactic acid production.

3.2 Results

3.2.2 Selection of the strain *Lactobacillus acidophilus* ATCC 43121 and its application to Brewers' Spent Grain conversion into lactic acid (Paper II)

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Research Article

Selection of the Strain *Lactobacillus acidophilus* ATCC 43121 and Its Application to Brewers' Spent Grain Conversion into Lactic Acid

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Six *Lactobacillus* strains were analyzed to select a bacterium for conversion of brewers' spent grain (BSG) into lactic acid. Among the investigated strains, *L. acidophilus* ATCC 43121 showed the highest yield of lactic acid production (16.1 g/L after 48 hours) when grown in a synthetic medium. It was then analyzed for its ability to grow on the hydrolysates obtained from BSG after acid-alkaline (AAT) or aqueous ammonia soaking (AAS) pretreatment. The lactic acid production by *L. acidophilus* ATCC 43121 through fermentation of the hydrolysate from AAS treated BSG was 96% higher than that from the AAT treated one, although similar yields of lactic acid per consumed glucose were achieved due to a higher (46%) glucose consumption by *L. acidophilus* ATCC 43121 in the AAS BSG hydrolysate. It is worth noting that adding yeast extract to the BSG hydrolysates increased both the yield of lactic acid per substrate consumed and the volumetric productivity. The best results were obtained by fermentation of AAS BSG hydrolysate supplemented by yeast extract, in which the strain produced 22.16 g/L of lactic acid (yield of 0.61 g/g), 27% higher than the value (17.49 g/L) obtained in the absence of a nitrogen source.

1. Introduction

Bioconversion of lignocellulosic residual biomass can make a significant contribution to the production of organic biochemicals [1]. Huge amounts of lignocellulosic wastes are produced yearly all around the world. They include agricultural residues, food farming wastes, green-grocer's wastes, tree pruning residues, and the organic and paper fractions of urban solid wastes. A wide range of high added value products, such as biofuels, organic acids, biopolymers, bioelectricity, and molecules, for the food and pharmaceutical industries [1] can be obtained by upgrading solid wastes through biotechnological processes.

One of the most important organic compounds is lactic acid, recognized as a GRAS (generally recognized as safe) compound by the US FDA (Food and Drug Administration), with many applications in food, cosmetics, and pharmaceutical and chemical industries [2]. Moreover, it has received a great deal of attention as a feedstock monomer for the production of PLA (polylactic acid), used as a biodegradable commodity plastic.

Microbial production of lactic acid from lignocellulosic wastes necessitates the pretreatment of lignocellulosic biomass to remove the barrier of lignin and expose the polysaccharides on the plant cell wall, the enzymatic saccharification of the polysaccharides with a (hemi)cellulolytic enzyme

cocktail, and the fermentation of the resulting sugars with lactic acid producing microorganisms [3].

Among the lignocellulosic wastes, BSG is considered as a valuable low-cost feedstock with considerable attractiveness for energy production. BSG represents the major by-product of the brewing industry. It accounts for about 85% of the total residues generated after the mashing and lautering processes [4] and is available in large quantities throughout the year. The chemical composition of BSG varies according to several factors such as the barley variety, the harvest time, and the malting and mashing conditions. BSG, as with any other lignocellulosic waste, is susceptible to transformation into a variety of different value-added products.

In this paper, six lactic acid bacteria (LAB) were compared for their ability to produce lactic acid in a synthetic growth medium. The most productive strain was investigated for its ability to produce lactic acid from the sugar mixture obtained by enzymatic hydrolysis of pretreated BSGs from two different geographical areas. The hydrolysates obtained by enzymatic hydrolysis of BSGs after two types of pretreatment, aqueous ammonia soaking and acid-alkaline pretreatment, were evaluated as substrate for the growth of the selected bacterial strain and the lactic acid production.

2. Materials and Methods

2.1. Chemical Pretreatments of Brewers' Spent Grains and Enzymatic Hydrolysis of Pretreated Materials. The brewers' spent grains provided by the brewery Bier Hoff Curitiba-PR (Brazil) (BSG 1) were subjected to acid-alkaline treatment (AAT). This consisted of a first treatment with 1.25% (v/v) H_2SO_4 in 1 : 8 (w/w) ratio at 120°C for 17 minutes [5]. The solid residue was washed with water until the pH was neutral, dried overnight at 50 ± 5°C, and mixed with 2% (v/v) NaOH in a 1 : 20 (w/w) ratio at 120°C for 90 minutes [6]. The cellulose pulp was washed with water until the pH was neutral, and it was dried overnight at 50 ± 5°C.

The brewer spent grains provided by the microbrewery Maneba (Striano, Naples, Italy) (BSG 2) were subjected to an aqueous ammonia soaking (AAS) treatment on a lab-scale as described by Maurelli et al. [7]. The biomass milled to a fine powder was suspended in 5% (v/v) aqueous ammonium hydroxide solution at a solid to liquid ratio of 1 : 10 and incubated at 70°C for 22 hours in screw-capped 25 mL bottles to reduce the evaporation. The solid residue, recovered by centrifugation at 8000 ×g, was extensively washed with water until reaching neutral pH and dried overnight at 50 ± 5°C.

The saccharification experiments were carried out in a total volume of 50 mL containing 50 mM sodium citrate buffer pH 5.5 plus enzyme cocktail (2.24% (v/v) cellulase and 1% (v/v) β-glucosidase, Novozymes) at 45°C, 60 ×g for 72 hours. To reach a comparable cellulose content in the pretreated lignocellulosic substrates subjected to enzymatic hydrolysis, the pretreated BSG samples, AAT BSG 1 and AAS BSG 2, were added at a solid loading of 8 and 16% (w/v), respectively. The liquid fraction was then separated from the solid residue by centrifugation.

2.2. Determination of Chemical Composition of Raw and Pretreated Materials. Carbohydrate compositions of the biomass samples untreated and pretreated were determined according to the method of Davis [8]. The polysaccharides were degraded into their corresponding monomers by a two-step acid hydrolysis. The samples were mixed with a 72% v/v H_2SO_4 solution at a solid loading of 10% and incubated at 30°C for 1 h; then, the mixtures diluted to 4% (v/v) H_2SO_4 with distilled water were incubated for 1 hour at 120°C. After filtration through 0.45 μm Teflon syringe filters (National Scientific, Lawrenceville, GA), the hydrolysis products were analyzed via high-performance liquid chromatography (HPLC) as described below. The acid insoluble lignin (Klason lignin) was determined, after total removal of the sugars by the weight of the dried residue.

2.3. Microorganisms and Cultivation Conditions for Screening in Synthetic Medium. The strains *Lactobacillus acidophilus* ATCC 53672, *L. acidophilus* ATCC 43121, *L. acidophilus* ATCC 4356, *L. lactis* INRA 18, *L. pentosus* NRRL B-227, and *L. plantarum* NRRL B-4496, all belonging to the strain collection of the Bioprocess and Biotechnology Division of the Department of "Engenharia de Bioprocessos e Biotecnologia" (Universidade Federal do Paraná, Brazil), were used in this study. Stock cultures were maintained at −20°C in 2 mL vials containing 25% (v/v) glycerol. The preinoculum was prepared by transferring 1 mL of stock culture cells to 25 mL tubes containing 10 mL of sterile Man, Rogosa, and Sharpe (MRS, Oxoid) culture medium with the following composition: 20 g/L glucose, 10 g/L peptone, 8 g/L meat extract, 4 g/L yeast extract, 2 g/L triammonium citrate, 2 g/L K_2HPO_4, 5 g/L $CH_3COONa{\cdot}3H_2O$, 0.2 g/L $MgSO_4{\cdot}7H_2O$, and 0.05 g/L $MnSO_4{\cdot}4H_2O$. The tubes were statically incubated for 24 hours at 37°C. Fifty microliters of this culture was then transferred to a MRS agar Petri dish supplemented with 1 g/L Aniline Blue (Sigma-Aldrich). One single blue lactic bacterial colony was transferred into 15 mL sterile MRS broth medium contained within a 25 mL test tube. The tubes were statically incubated at 37°C for 48 hours. After incubation, 10 mL of this culture was transferred into a 250 mL Erlenmeyer flask containing 100 mL of MRS fermentation broth. The flasks were incubated at 37°C and 60 ×g for 96 hours during which time samples were withdrawn every 24 hours and subjected to analytical testing.

2.4. Inoculum and Fermentation Conditions for Cultivation in BSG Hydrolysate. The strain selected as the best producer of lactic acid in the primary screening was grown in 25 mL tubes containing 10 mL MRS broth. After 24 hours of static incubation at 37°C, 1 mL of this culture was transferred to a new tube with 10 mL MRS broth and incubated at the same conditions. Then, the cells were harvested by centrifugation (3.300 ×g for 15 minutes) and resuspended in distilled sterilized water. The glucose concentration of BSG hydrolysate was adjusted to 50 g/L with deionized water, while the pH was adjusted to a value of 6.0 by adding 5 M NaOH. The fermentation medium, with or without the addition of 1.25% (w/v) yeast extract, was then sterilized at 120°C for 15 minutes. Fermentations were

performed in 250 Erlenmeyer flask containing 50 mL sterilized hydrolysate inoculated with 10% (v/v) of cell suspension. The flasks were incubated at 37°C and 60 ×g for 96 hours. All of the assays were performed in duplicate.

2.5. Analytical Methods. Samples taken during the fermentations at 24, 48, 72, and 96 hours were centrifuged at 12.800 g for 15 minutes. The resulting supernatant was used for measurement of glucose and lactic acid concentrations by high-performance liquid chromatography (HPLC). The HPLC analyses were performed in a Shimadzu LC-10-AD equipped with a C-RSA Integrator Chromatopac Chromatography Unit (Shimadzu) set to 210 nm (300 mm × 7.8 mm) column HPX-87-4 (Aminex) and a refractive index detector. The samples were diluted with deionized water, filtered through 0.2 μm cellulose acetate filters (Sartorius Biolab Products), and then injected into the chromatograph. The conditions of chromatography involved a column temperature of 60°C, an injection volume of 50 μL, and the elution with a mobile phase of 5 mM sulfuric acid at a flow rate of 0.6 mL/min.

The concentration of these compounds was calculated through calibration curves obtained using standard solutions. Also the sugars released from untreated and pretreated biomasses prepared as above described were estimated. Cell concentration was obtained by measuring the optical density ($OD_{600\,nm}$) in a spectrophotometer SP-2000 (Spectrum). Samples were diluted to a reading range of 0.05–0.5 OD units, and the OD values were correlated with the cell concentration (g/L) by means of a standard calibration curve previously established. The calibration curve for cell concentration and absorbance showed a linear relationship. One unit of optical density at 600 nm corresponded to approximately 0.6 g dry-cell weight/L.

2.6. Fermentative Parameters. The ratio of lactic acid produced to glucose consumed (g/g), the ratio of lactic acid (g/L) to fermentation time (h), the ratio of lactic acid to dry-cell weight (g/g), and the ratio of lactic acid yield per substrate consumed to the maximum theoretical value (1 g/g) were monitored during the fermentation of *Lactobacillus acidophilus* ATCC 43121.

3. Results and Discussion

3.1. Screening of Lactic Acid Bacteria for Lactic Acid Production in Synthetic Medium. The lactic acid bacteria (LAB) *Lactobacillus acidophilus* ATCC 53672, *L. acidophilus* ATCC 43121, *L. acidophilus* ATCC 4356, *L. lactis* INRA 18, *L. pentosus* NRRL B-227, and *L. plantarum* NRRL B-4496 were compared for their lactic acid production ability in a synthetic medium. Since LAB have complex nutrient requirements, due to their limited ability to synthesize B-vitamins and amino acids [9], the Man, Rogosa, and Sharpe (MRS) broth containing minerals, B-vitamins, amino acids, fatty acids, purines, and pyrimidines was used as the fermentation medium. Time courses of dry biomass, glucose, and lactic acid concentration were monitored during batch fermentations of the six *Lactobacillus* strains in MRS broth (Figures 1(a)–1(f)).

The analyzed *Lactobacillus* strains showed significant differences in the fermentation performances, for example, cell growth and productivity. The maximum biomass varied from 1.9 ± 0.5 g/L for *L. acidophilus* ATCC 4356 (Figure 1(c)) to 4.56±0.5 g/L for *L. lactis* INRA 18 (Figure 1(d)). Regarding the volumetric productivity, the values varied from a minimum of 0.22 g/L h and a maximum of 0.50 g/L h, reached by the strains *L. acidophilus* ATCC 53672 (Figure 1(a)) and *L. lactis* INRA 18 (Figure 1(d)), respectively. The maximum value of lactic acid production varied from 8.3 ± 0.6 g/L for *L. pentosus* NRRL B-227 (Figure 1(e)) to 16.1 ± 0.07 g/L for *L. acidophilus* ATCC 43121 (Figure 1(b)). *L. acidophilus* ATCC 43121 (Figure 1(b)), *L. acidophilus* ATCC 4356 (Figure 1(c)), and *L. plantarum* NRRL B-4496 (Figure 1(f)) showed the highest fermentation yield, with a maximum value of lactic acid production of 16.1 ± 0.07 g/L (after 48 hours), 15.84 ± 0.05 g/L (after 48 hours), and 15.66 g/L ± 0.04 (after 72 hours), respectively. The maximum $Y_{P/S}$ value varied from a minimum of 0.52 g/g for the strain *L. pentosus* NRRL B-227 to a maximum of 0.99 g/g for *L. acidophilus* ATCC 43121, followed by the strains *L. acidophilus* ATCC 53672, *L. plantarum* NRRL B-4496, *L. acidophilus* ATCC 4356, and *L. lactis* INRA 18 with $Y_{P/S}$ of 0.98 g/g, 0.96 g/g, 0.95 g/g, and 0.70 g/g, respectively.

L. acidophilus ATCC 43121, showing a volumetric productivity value of 0.34 g/L h and the highest lactic acid production, was selected for lactic acid production from the sugar mixture obtained by enzymatic hydrolysis of chemically pretreated BSG.

3.2. Chemical Pretreatments of Brewers' Spent Grains. BSGs from two different geographical areas were investigated as substrate for *L. acidophilus* ATCC 43121 growth and lactic acid production. The pretreatment of BSGs was performed by adopting two chemical methods. The biomass obtained from the brewery Bier Hoff Curitiba-PR Brazil, indicated as BSG 1, was subjected to an acid-alkaline treatment (AAT), whilst an alkaline pretreatment was conducted on the BSG from microbrewery Maneba Striano, Naples, Italy (BSG 2).

3.3. Acid-Alkaline Pretreatment. The acid-alkaline treatment (AAT) performed on the biomass obtained from the brewery Bier Hoff Curitiba-PR Brazil (BSG 1) involved two sequential steps accomplished through an acid impregnation followed by a biomass soaking in an alkaline solution. The procedure and operative conditions adopted were those tested by Mussatto and Roberto [5]. The BSG 1 was treated with 1.25% (v/v) H_2SO_4 to solubilize the hemicellulosic fraction. The initial removal of the hemicellulose shows the advantage of increasing the porosity of the material that facilitates the diffusion and impregnation of sodium hydroxide into the material sample, thus enhancing the soda pulping and the pulp uniformity. Moreover, since the hemicellulose is a valuable source of xylose, its recovery allows the sugar exploitation to produce value-added products such as xylitol [5] or ethanol [10]. After acid hydrolysis, a soda pulping pretreatment with 2% (v/v) NaOH was performed to liberate cellulose fibers from lignin [6].

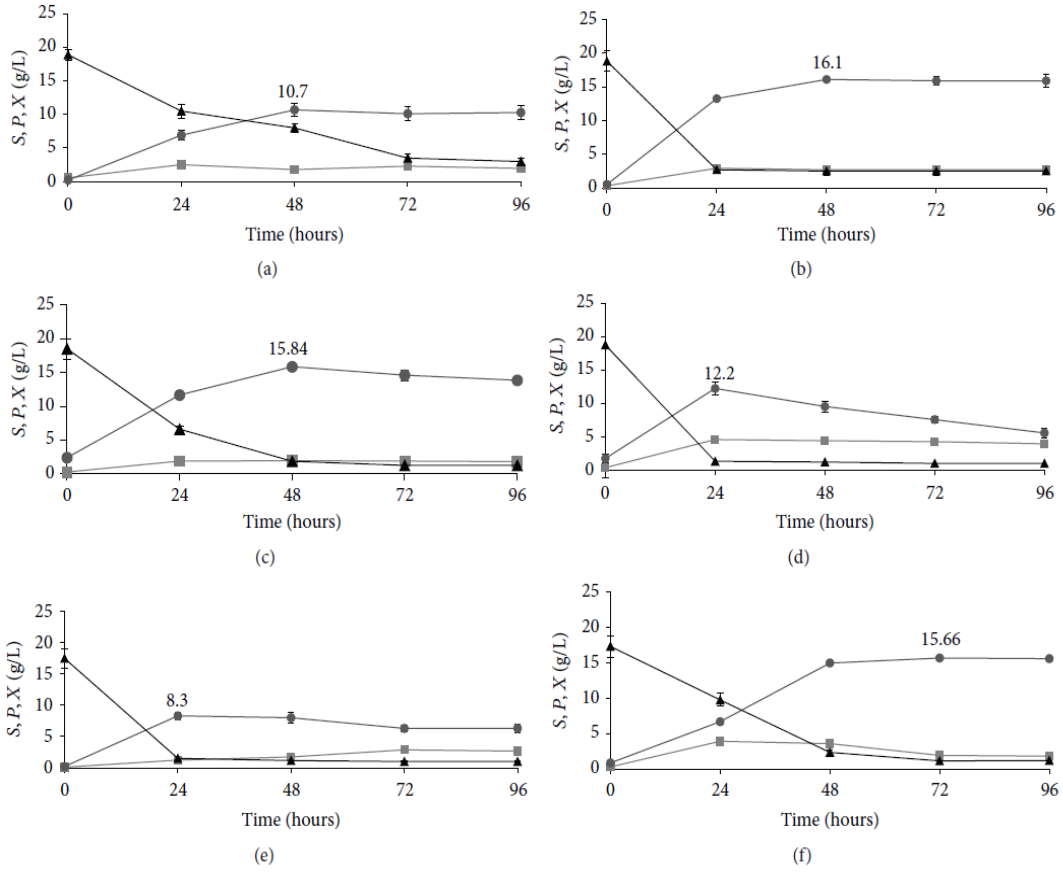

FIGURE 1: Time courses of glucose concentration (S, △), dry biomass concentration (X, □), and lactic acid concentration (P, o) during fermentation in synthetic medium of the *Lactobacillus* sp. strains: (a) *L. acidophilus* ATCC 53672, (b) *L. acidophilus* ATCC 43121, (c) *L. acidophilus* ATCC 4356, (d) *L. lactis* INRA 18, (e) *L. pentosus* NRRL b-227, and (f) *L. plantarum* NRRL b-4496. Data represent the mean of three independent experiments.

TABLE 1: Chemical composition (% w/w) of brewer's spent grains in the original form and as a cellulose pulp recovered by acid-alkaline and aqueous ammonia soaking pretreatments.

	Solid composition			
	Cellulose % (w/w)	Hemicellulose % (w/w)	Lignin % (w/w)	Others (ash, protein, and extractives) % (w/w)
Untreated BSG 1[a]	14.42	34.21	3.93	47.43
Untreated BSG 2[b]	27.50	28.80	12.80	30.9
AAT[c] BSG 1	86.49	3.87	2.31	7.33
AAS[d] BSG 2	43.20	38.85	4.83	13.12

[a]BSG from the brewery Bier Hoff Curitiba-PR (Brazil).
[b]BSG from the microbrewery Maneba (Striano, Naples, Italy).
[c]BSG after acid-alkaline treatment (AAT).
[d]BSG after the aqueous ammonia soaking (AAS) treatment.

Chemical analyses of the untreated and pretreated BSG 1 were performed to determine the macromolecular composition, and the results are reported in Table 1.

After pretreatment, the total solid biomass recovered was 7.89% (w/w). The hemicellulose was the main fraction removed (88.68% (w/w)), whilst a loss of 41.22% (w/w) and 84.54% (w/w) was obtained for lignin and other materials (ash, protein, and extractives), respectively. The cellulose content was 86.49% (w/w), corresponding to the main fraction of the pretreated BSG. However, if we considered that, after

the pretreatment, the mass recovered from each 100 g of the original BSG was 7.89 g, the cellulose loss, calculated by the percentage of each fraction, was 52.65%.

3.4. Aqueous Ammonia Soaking Pretreatment. An alkaline pretreatment was conducted on the BSG 2 from microbrewery Maneba Striano, Naples, Italy, by soaking the lignocellulosic material in 5% ammonium hydroxide solution at 70°C for 22 h. As reported in literature, the alkali pretreatment is one of the most widely used methods to enhance the lignocellulosic biomass digestibility. One of the major advantages of this method is the limited hydrolysis of the (hemi)cellulosic fraction which prevents the production of sugar degradation products which inhibit the microbial fermentation, such as furfural [11]. The principal effect of the alkaline pretreatment is the lignin removal from the biomass through reactions of solvation and saponification of the intermolecular ester bonds among lignin, hemicellulose, and cellulose. Moreover, alkaline pretreatment causing the biomass swelling increases the lignocellulosic material porosity and reduces the polymerization degree and the crystallinity of the cellulose [12]. This in conjunction with the disruption of the lignin structure makes the cellulose more susceptible to enzymatic hydrolysis [11]. Soaking in aqueous ammonia solutions (AAS) is a common alkaline pretreatment that reduces lignin content and removes some hemicellulose while decrystallizing cellulose. At the mild conditions adopted in AAS, the level of end-groups dissolution, "peeling," of the different polysaccharide chains is extremely limited thus avoiding carbohydrates loss and conversion of the lost sugars to compounds (e.g., furfural) with inhibitory effect on the fermentation process [13].

Chemical analyses of the untreated and AAS pretreated BSG 2 were performed to determine their macromolecular composition (Table 1). After pretreatment, the total solid biomass recovered was 60% (w/w). On the basis of the % composition of the material samples, a big fraction of lignin was removed (62.26%) whilst the cellulose and hemicellulose underwent an increase of 57.09 and 34.89%, respectively. With respect to the mass recovered from 100 g of the original BSG, calculated by the percentage of each fraction in 60 g of the pretreated material (total recovered mass after the pretreatment), it is evident that the highest losses were reported for the lignin fraction (77.3%) whilst only 19 and 5.8% of the hemicellulose and cellulose were removed, respectively. In agreement with the literature on the alkaline pretreatments, our results indicate that ammonium hydroxide pretreatment causes substantial lignin degradation whilst preserving most of the carbohydrates [14]. In particular, most of the cellulose was preserved probably due to the physical protection from lignin and hemicellulose. On the other hand, the heterostructures of xylan branched with short lateral chains contributed to the higher hemicelluloses hydrolysis [15].

3.5. Saccharification of the Pretreated BSGs by a Cocktail of Commercial Enzymes. The pretreated BSG samples were saccharified with commercial cellulase and β-glucosidase from Novozymes. The ratio of enzymes in the cocktail used for the saccharification was defined based on the composition of pretreated BSG samples reported in Table 1. The enzymatic saccharification of AAT BSG 1 and AAS BSG 2 generated hydrolysates containing 75 and 60 g/L of glucose, respectively. At the end of the reaction, 97 and 86.8% of efficiency of cellulose conversion into glucose were achieved for AAT BSG 1 and AAS BSG 2, respectively. These conversion yields are higher than that of 72% obtained by Mussatto et al. [16] on BSG pretreated with a similar acid-alkaline pretreatment. It can be observed that although the cellulose amounts (~90% w/w) in the biomasses employed in our saccharification experiments were comparable to those described by Mussatto et al. [16], the lignin ones were quite different. Thus, it is possible that the bigger lignin content (8.2% w/w) of the pretreated BSG utilized by Mussatto et al. [16] decreased the enzymes accessibility to the cellulose leading to the lower conversion yield.

3.6. Lactic Acid Production Using BSG Hydrolysates after Acid-Alkaline or Ammonia Soaking Pretreatment as Fermentation Media. The hydrolysates obtained from the saccharification of the differently pretreated BSGs were used to prepare the fermentation media for the lactic acid production by *Lactobacillus acidophilus* ATCC 43121, the strain previously selected among the analyzed LAB. The BSG hydrolysates, without adding any additional nutrients, and the BSG hydrolysates supplemented with the addition of 1.25% (w/v) yeast extract were adjusted to pH 6.0 and glucose concentration of 50 g/L by adding sterile water. It was observed that glucose consumption and lactic acid production occurred in all tested fermentation media (Figures 2(a)–2(d), Table 2).

The maximum value of lactic acid production was obtained from the AAS BSG 2 hydrolysate in the presence of yeast extract and it was 80% higher than that from the AAT BSG 1. Even in the absence of yeast extract, AAS BSG 2 hydrolysate allowed achieving a (around 96%) higher lactic acid production than the AAT BSG 1 hydrolysate. In any tested condition, the maximum production was observed after 48 hours.

The higher lactic acid production from AAS BSG 2 hydrolysate generates higher values of Q_P and $Y_{P/X}$ than the AAT BSG 1 hydrolysate (Table 2).

It is worth noting that the glucose consumption increases in the AAS BSG 2 hydrolysate, resulting in being 46% higher than that measured in the AAT BSG 1 hydrolysate (Table 2), thus giving similar values of $Y_{P/S}$ and fermentation efficiency for the two pretreatments. Moreover, in the AAT BSG 1 hydrolysate, *L. acidophilus* ATCC 43121 remained in a stationary phase until the end of fermentation whilst, in AAS BSG 2, the bacterium entered in early death phase at 72 h, which can be due to the poor residual glucose (Figures 2(a)–2(d)).

Using the hydrolysate from AAS BSG 2 allowed achieving higher biomass concentration than the AAT BSG 1, both in the presence and in the absence of yeast extract.

It is worthy of noting that the yield of lactic acid per substrate consumed ($Y_{P/S}$) obtained using *L. acidophilus* ATCC 43121 grown in the BSG hydrolysate, without adding any additional element, was 0.48 g/g (from AAT BSG 1 hydrolysate)

Table 2: Fermentative parameters of lactic acid production of *Lactobacillus acidophilus* ATCC 43121 by using hydrolysates of brewer's spent grain from the brewery Bier Hoff Curitiba-PR (Brazil) (BSG 1) after acid-alkaline treatment (AAT) and hydrolysates of brewer's spent grain from the microbrewery Maneba (Striano, Naples, Italy) (BSG 2) after aqueous ammonia soaking (AAS) treatment.

	Glucose consumption (g/L)	Lactic acid (g/L)	$Y_{P/S}$ [a] (g/g)	$Y_{P/X}$ [b] (g/g)	Q_P [c] (g/L h)	η [d] (%)
AAT BSG 1 hydrolysate	18.20	8.90	0.48	4.45	0.09	48
AAT BSG 1 hydrolysate + yeast extract	20.50	12.26	0.60	4.69	0.12	60
AAS BSG 2 hydrolysate	33.50	17.49	0.52	7.64	0.18	52
AAS BSG 2 hydrolysate + yeast extract	37.40	22.16	0.61	5.61	0.31	61

[a]g-Lactic acid produced/g-glucose consumed.
[b]g-Lactic acid/g-dry-cell weight.
[c](g/L)-Lactic acid/(h) fermentation time.
[d]$Y_{P/S}$/maximum theoretical value (1 g/g).

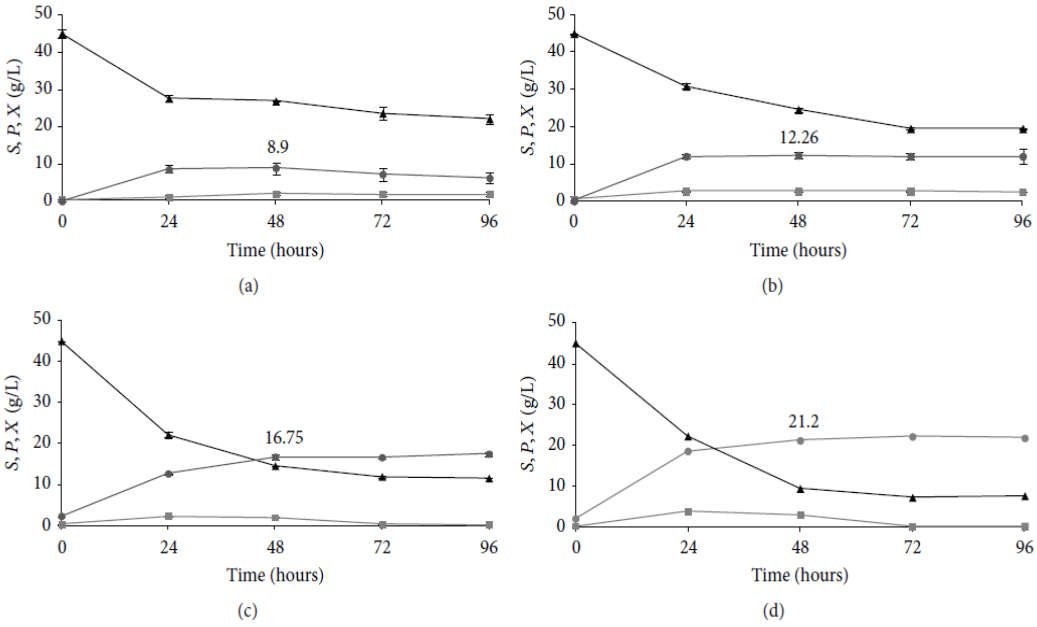

Figure 2: Time courses of glucose concentration ($S, \triangle$), dry biomass concentration ($X, \square$), and lactic acid concentration ($P, \circ$) during fermentation of *Lactobacillus acidophilus* ATCC 43121 in (a) acid-alkaline treated brewer's spent grain 1 hydrolysate, (b) acid-alkaline treated brewer's spent grain 1 hydrolysate + yeast extract, (c) aqueous ammonia soaking treated brewer's spent grain 2 hydrolysate, and (d) aqueous ammonia soaking treated brewer's spent grain 2 hydrolysate + yeast extract. All values are media of three replications.

and 0.52 g/g (from AAS BSG 2 hydrolysate). This demonstrates that the BSG hydrolysate is an appropriate substrate for lactic acid production. Since the lactic acid is considered a relatively cheap product, the use of expensive carbon sources, such as glucose or starch, is not economical. Less expensive sources, like agroindustrial residues, are attractive alternatives.

The maximum lactic acid production levels shown in this study were higher than those obtained in the other few studies so far reported on the use of hydrolysates from agricultural residues as fermentation medium. The results of these studies are summarized in Table 3. In the best conditions, the values obtained in our studies for lactic acid production were higher than those reported by Jawad et al. [17], McCaskey et al. [18], and Mussatto et al. [19] and just a little lower than those obtained by Ali et al. [20]. As for the yields $Y_{P/S}$, the maximum value obtained in our work was 14% lower than the maximum values previously reported [18, 19] indicating higher nutritional needs of *L. acidophilus* ATCC 43121 than the microbes adopted in the other studies. When the strain *L. acidophilus* ATCC 43121 was grown in the BSG hydrolysate supplemented with 1.25% (w/v) yeast extract, the yield of lactic acid increased from 0.48 g/g to 0.60 g/g (65% efficiency) for AAT BSG 1 hydrolysate and from 0.52 g/g to 0.61 g/g (65% efficiency) for AAS BSG 2 (Table 2).

Also the volumetric productivity (Q_p) of lactic acid by *L. acidophilus* ATCC 43121 was higher in the BSG hydrolysate supplemented with yeast extract. Within 48 hours of fermentation, the cells produced a maximum value of 12.26 g/L of lactic acid from AAT BSG 1 hydrolysate and 22.16 g/L of

Table 3: Lactic acid production from agricultural residue hydrolysates.

Waste type	Waste pretreatment	Enzymatic hydrolysis	Fermentation conditions	Yield of glucose after enzymatic hydrolysis	Microorganism used in fermentation step	Production levels of lactic acid	Yield of lactic acid per glucose consumed	References
Brewer's spent grain	1.25% (v/v) sulfuric acid solution in a 1 : 8 g : g solid : liquid ratio, at 120°C for 17 minutes; 2% (v/v) sodium hydroxide solution in a 1 : 20 g : g solid : liquid ratio, at 120°C for 90 minutes	2.24% (v/v) cellulase (Novozymes) and 1% (v/v) β-glucosidase (Novozymes) using 8% (w/v) substrate, at 45°C and 120 rpm for 72 hours	Inoculum with 10% (v/v) of cell suspension, at 37°C and 60 ×g	75.0 g/L	*Lactobacillus acidophilus* ATCC 43121	8.9 g/L from AAT BSG 1 12.26 g/L from AAT BSG 1 + yeast extract 17.49 g/L from AAS BSG 2 22.16 g/L from AAS BSG 2 + yeast extract	0.48 g/g from AAT BSG 1 0.6 g/g from AAT BSG 1 + yeast extract 0.52 g/g from AAS BSG 2 0.61 g/g from AAS BSG 2 + yeast extract	This study
Mango peel	—	—	Coupling of the microbial hydrolysis and fermentation of the carbohydrate substrate into a single step at 35°C under static incubation conditions	—	Consortium of indigenous microorganisms of mango peels	17.48 g/L	—	[17]
Municipal solid waste (MSW)	2% (v/v) of H_2SO_4 (1 : 10 solid-to-liquid ratio) at 124°C for 15 minutes	—	Optimum conditions: initial pH 7.6, 1% v/v inoculum, and 5% (w/v) calcium carbonate buffer at 32°C	41.3 g/L	*Lactobacillus arabinosus* B-787, *L. arabinosus* B-788, *L. arabinosus* B-813, *L. arabinosus* B-531, *L. pentosus* B-277, *L. pentosus* B-473, *L. plantarum* USDA 422, and *L. xylosus* B-4449	~17–19 g/L excepted for *L. xylosus* B-4449 ~6 g/L	From 0.52 g/g to 0.73 g/g	[18]
Brewer's spent grain	1.25% (v/v) sulfuric acid solution in a 1 : 8 g : g solid : liquid ratio, at 120°C for 17 minutes; 2% (v/v) sodium hydroxide solution in a 1 : 20 g : g solid : liquid ratio, at 120°C for 90 minutes	Celluclast 1.5 L from *Trichoderma reesei* (Novozymes, Denmark)	Inoculated with an initial cell concentration of 1.0 g/L; statical incubation at 37°C	50.0 g/L	*Lactobacillus delbrueckii*	7.87 g/L using nonsupplemented BSG hydrolysate ~9 g/L using BSG hydrolysate supplemented with yeast extract	0.7 g/g	[19]
Corn cobs	5, 10, and 15% (v/v) of H_2SO_4 autoclaved at different temperatures for different intervals of time	—	Optimum conditions: 40°C, pH 5-6	4.0 (% w/w)	*Lactobacillus delbrueckii*	25.62 g/L	—	[20]

lactic acid from the AAS BSG 2 hydrolysate with a volumetric productivity of 0.12 g/L h and 0.31 g/L h, respectively. These Q_P values are higher when compared with 0.09 g/L h and 0.18 g/L h produced in the AAT BSG 1 hydrolysate and the AAS BSG 2 without yeast extract addition, respectively (Table 2).

It is possible that the addition of yeast extract is necessary to reach an optimal C/N ratio for the lactic acid production by the *Lactobacillus* strain. These results are in agreement with those of many authors, showing the positive effect of BSG hydrolysate supplementation with additional nutrients, like yeast extract or vitamins, on lactic acid production. Yeast extract is the most commonly used nitrogen source, which provides the vitamin B complex content in addition to organic nitrogen to lactic acid bacteria.

Since the addition of yeast extract is disadvantageous from an economical point of view, more research will be needed to discover strains with lower nitrogen sources requests.

It was found that the maximum lactic acid productivity of the strain *L. casei* in a synthetic medium increased concomitantly with the initial yeast extract concentration. None of the other nitrogen sources tested gave lactic acid concentrations as high as that for yeast extract during 48 hours of fermentation [21].

Mussatto et al. [19] showed that the addition of 0.5% yeast extract enhanced the lactic acid production by *Lactobacillus delbrueckii*, reaching a concentration of ~9.0 g/L with a lactic acid yield ($Y_{P/S}$) of 0.7 g/g. This is a higher value than that obtained from nonsupplemented hydrolysate (7.87 g/L). When fermented in BSG hydrolysate after AAS pretreatment without any nutrient supplementation, the *L. acidophilus* ATCC 43121 strain used in this study reached a lactic acid concentration of 17.49 g/L, a value higher than the one (~9.0 g/L) obtained by *L. delbrueckii* in the BSG hydrolysate supplemented with yeast extract [19]. Moreover, when the strain *L. acidophilus* ATCC 43121 was grown in the AAS BSG 2 hydrolysate supplemented with 1.25% yeast extract, a maximum lactic acid concentration of 22.16 g/L was reached. This concentration is 2.5-fold higher than that obtained by *Lactobacillus delbrueckii* in the presence of 0.5% yeast extract [19], which represents the main achievement of our study in comparison with the work performed on *Lactobacillus delbrueckii*.

4. Conclusions

The strain *Lactobacillus acidophilus* ATCC 43121 selected for its higher lactic acid production in a synthetic medium was analyzed for its ability to produce lactic acid from BSG hydrolysate. The results indicated that the produced hydrolysates from ammonia soaking treated BSG 2, provided by the microbrewery Maneba (Striano, Naples, Italy), and from the acid-alkaline treated BSG 1, provided by the brewery Bier Hoff Curitiba-PR (Brazil), are suitable substrates for the growth of the strain *L. acidophilus* LPB-04 and the production of lactic acid. Moreover, it was demonstrated that the bioconversion of glucose into lactic acid was positively affected by the presence of yeast extract. The maximum value of lactic acid production (22.16 g/L) was obtained from the AAS BSG 2 hydrolysate in the presence of yeast extract. The produced levels of lactic acid were comparable or higher in comparison with those obtained from hydrolysates of other agricultural residues. This result is in keeping with the many researches focused on the valorization of BSG in order to recover beneficial and valuable compounds, usable in nutrition or combustion field. Due to its composition and availability, in the last years, different methods to remove the water content, avoiding the BSG degradation [22–24], and to separate each component of it have been proposed [25, 26], taking different economical and ecofriendly advantages into account.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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References

[1] R. Liguori, A. Amore, and V. Faraco, "Waste valorization by biotechnological conversion into added value products," *Applied Microbiology and Biotechnology*, vol. 97, no. 14, pp. 6129–6147, 2013.

[2] R. Datta, S. P. Tsai, P. Bonsignore, S. H. Moon, and J. R. Frank, "Technological and economic potential of poly(lactic acid) and lactic acid derivatives," *FEMS Microbiology Reviews*, vol. 16, no. 2-3, pp. 221–231, 1995.

[3] T. Ghaffar, M. Irshad, Z. Anwar et al., "Recent trends in lactic acid biotechnology: a brief review on production to purification," *Journal of Radiation Research and Applied Sciences*, vol. 7, no. 2, pp. 222–229, 2014.

[4] S. I. Mussatto, G. Dragone, and I. C. Roberto, "Brewers' spent grain: generation, characteristics and potential applications," *Journal of Cereal Science*, vol. 43, no. 1, pp. 1–14, 2006.

[5] S. I. Mussatto and I. C. Roberto, "Acid hydrolysis and fermentation of brewer's spent grain to produce xylitol," *Journal of the Science of Food and Agriculture*, vol. 85, no. 14, pp. 2453–2460, 2005.

[6] S. I. Mussatto, G. Dragone, G. J. M. Rocha, and I. C. Roberto, "Optimum operating conditions for brewer's spent grain soda pulping," *Carbohydrate Polymers*, vol. 64, no. 1, pp. 22–28, 2006.

[7] L. Maurelli, E. Ionata, F. La Cara, and A. Morana, "Chestnut shell as unexploited source of fermentable sugars: effect of different pretreatment methods on enzymatic saccharification," *Applied Biochemistry and Biotechnology*, vol. 170, no. 5, pp. 1104–1118, 2013.

[8] M. W. Davis, "A rapid modified method for compositional carbohydrate analysis of lignocellulosics by high pH anion-exchange chromatography with pulsed amperometric detection (HPAEC/PAD)," *Journal of Wood Chemistry and Technology*, vol. 18, no. 2, pp. 235–352, 1998.

[9] T. Morishita, Y. Deguchi, M. Yajima, T. Sakurai, and T. Yura, "Multiple nutritional requirements of lactobacilli: genetic lesions affecting amino acid biosynthetic pathways," *Journal of Bacteriology*, vol. 148, no. 1, pp. 64–71, 1981.

[10] M. D. Ferrari, E. Neirotti, C. Albornoz, and E. Saucedo, "Ethanol production from eucalyptus wood hemicellulose hydrolysate by *Pichia stipitis*," *Biotechnology and Bioengineering*, vol. 40, no. 7, pp. 753–759, 1992.

[11] J. Singh, M. Suhag, and A. Dhaka, "Augmented digestion of lignocellulose by steam explosion, acid and alkaline pretreatment methods: a review," *Carbohydrate Polymers*, vol. 117, pp. 624–631, 2015.

[12] B. Joshi, M. R. Bhatt, D. Sharma, J. Joshi, R. Malla, and L. Sreerama, "Lignocellulosic ethanol production: current practices and recent developments," *Biotechnology and Molecular Biology Review*, vol. 6, no. 8, pp. 172–182, 2011.

[13] M. J. Taherzadeh and K. Karimi, "Pretreatment of lignocellulosic wastes to improve ethanol and biogas production: a review," *International Journal of Molecular Sciences*, vol. 9, no. 9, pp. 1621–1651, 2008.

[14] F. M. Gírio, C. Fonseca, F. Carvalheiro, L. C. Duarte, S. Marques, and R. Bogel-Łukasik, "Hemicelluloses for fuel ethanol: a review," *Bioresource Technology*, vol. 101, no. 13, pp. 4775–4800, 2010.

[15] A. T. W. M. Hendriks and G. Zeeman, "Pretreatments to enhance the digestibility of lignocellulosic biomass," *Bioresource Technology*, vol. 100, no. 1, pp. 10–18, 2009.

[16] S. I. Mussatto, M. Fernandes, G. Dragone, I. M. Mancilha, and I. C. Roberto, "Brewer's spent grain as raw material for lactic acid production by *Lactobacillus delbrueckii*," *Biotechnology Letters*, vol. 29, no. 12, pp. 1973–1976, 2007.

[17] A. H. Jawad, A. F. M. Alkarkhi, O. C. Jason, A. M. Easa, and N. A. Nik Norulaini, "Production of the lactic acid from mango peel waste—factorial experiment," *Journal of King Saud University—Science*, vol. 25, no. 1, pp. 39–45, 2013.

[18] T. A. McCaskey, S. D. Zhou, S. N. Britt, and R. Strickland, "Bioconversion of municipal solid waste to lactic acid by *Lactobacillus* species," *Applied Biochemistry and Biotechnology*, vol. 45-46, no. 1, pp. 555–568, 1994.

[19] S. I. Mussatto, M. Fernandes, I. M. Mancilha, and I. C. Roberto, "Effects of medium supplementation and pH control on lactic acid production from Brewer's spent grain," *Biochemical Engineering Journal*, vol. 40, no. 3, pp. 437–444, 2008.

[20] Z. Ali, F. M. Anjum, and T. Zahoor, "Production of lactic acid from corn cobs hydrolysate through fermentation by *Lactobaccillus delbrukii*," *African Journal of Biotechnology*, vol. 8, no. 17, pp. 4175–4178, 2009.

[21] I.-K. Yoo, H. N. Chang, E. G. Lee, Y. K. Chang, and S.-H. Moon, "Effect of B vitamin supplementation on lactic acid production by *Lactobacillus casei*," *Journal of Fermentation and Bioengineering*, vol. 84, no. 2, pp. 172–175, 1997.

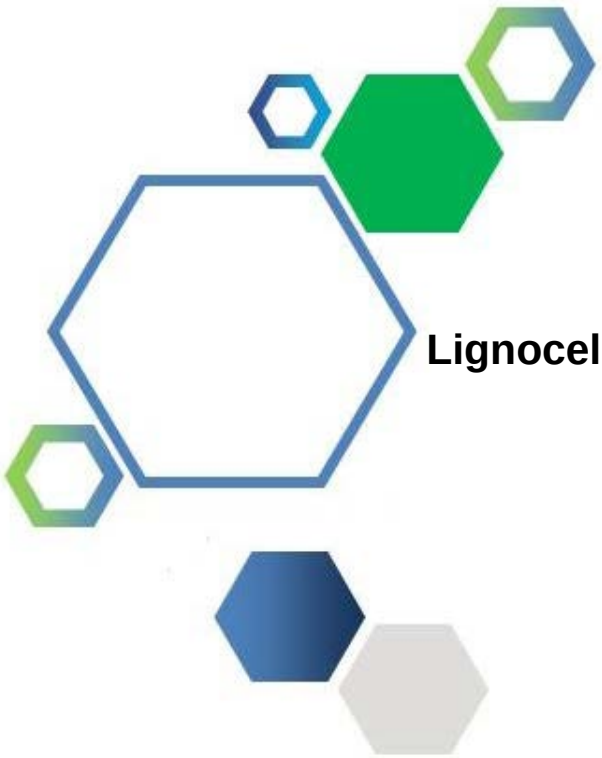
[22] F. Carvalheiro, M. P. Esteves, J. C. Parajó, H. Pereira, and F. M. Gírio, "Production of oligosaccharides by autohydrolysis of brewery's spent grain," *Bioresource Technology*, vol. 91, no. 1, pp. 93–100, 2004.

[23] G. Mandalari, C. B. Faulds, A. I. Sancho et al., "Fractionation and characterisation of arabinoxylans from brewers' spent grain and wheat bran," *Journal of Cereal Science*, vol. 42, no. 2, pp. 205–212, 2005.

[24] D.-S. Tang, G.-M. Yin, Y.-Z. He et al., "Recovery of protein from brewer's spent grain by ultrafiltration," *Biochemical Engineering Journal*, vol. 48, no. 1, pp. 1–5, 2009.

[25] E. I. El-Shafey, M. L. F. Gameiro, P. F. M. Correia, and J. M. R. de Carvalho, "Dewatering of brewer's spent grain using a membrane filter press: a pilot plant study," *Separation Science and Technology*, vol. 39, no. 14, pp. 3237–3261, 2004.

[26] M. P. Fernández, J. F. Rodriguez, M. T. García, A. de Lucas, and I. Gracia, "Application of supercritical fluid extraction to brewer's spent grain management," *Industrial and Engineering Chemistry Research*, vol. 47, no. 5, pp. 1614–1619, 2008.



Chapter IV

Lignocellulosic waste conversion for ethanol production

4.1 Introduction

Bioethanol has been recognized as a good alternative to petroleum-derived transportation fuels, with several known advantages, such as high octane number, low cetane number and high heat of vaporization [13]. The use of bioethanol for fuel was widespread in Brazil, United States and Europe until the early 1900s; however, since its production became more expensive than that of petroleum based fuel, the bioethanol's potential was ignored until the 1970s, when the oil crisis moved the world's interest towards the use of new renewable resources [14]. In the last decade, bioethanol production has increased rapidly because many countries have issued several "Fuel Ethanol Programs", aimed at mandating the use of ethanol blends through tax incentives or exemption [15]; this leads toward reducing oil imports, increasing rural economies along with improving the quality of air. In 2014, global bioethanol production churned out a record of 14.3 billion gallons, eclipsing the previous record of 13.9 billion gallons set in 2011. The United States is the leading producer, accounting for nearly 60 % of the global output. Brazil, which produced roughly 6.2 billion gallons, was responsible for about 25 % of world production, while the European Union followed with 6 % [16].

Generally, bioethanol can be mainly classified into "first-generation" and "second generation", where the main difference is the characteristic of the feedstock used. First generation bioethanol is made from edible sugars, grains or seeds, through a simple processing consisting of sugar extraction (e.g. through crushing), followed by fermentation and distillation. Brazil utilizes sugarcane for bioethanol production while the United States and Europe mainly use starch from corn, and from wheat and barley, respectively. However, many concerns are still associated to first generation bioethanol such as feedstock insufficiency related to the increased demand; food *versus* fuel conflict, due to the use of edible material for fermentable sugars recovery [17]. To overcome this bottleneck, growing attention has been focused on the production of second generation bioethanol, that is produced from non edible, renewable and worldwide available lignocellulosic resources. They include agricultural residues, food farming wastes, green-grocer's wastes, tree pruning residues and organic and paper fraction of urban solid wastes. Besides its numerous advantages, bioethanol production process from lignocellulosic crops is more difficult compared to sugar crops because expensive additional steps, consisting of pretreatment and hydrolysis, are required due to the complex structure of the feedstock. A pretreatment step allow to remove the barrier of lignin and expose the polysaccharides, that can be subsequently enzymatically hydrolyzed into fermentable monosaccharides [129]. Thus, many efforts are still needed to make the second generation ethanol production more eco-friendly and cost-effective.

This chapter describes the studies concerning the conversion of Brewers' Spent Grain (BSG) into ethanol through yeast strains fermentation. These activities were carried out in the laboratory of Dr. Carlos Ricardo Soccol at the Department of "Engenharia de Bioprocessos e Biotecnologia" (Universidade Federal do Paraná, Brazil) in the frame of the project BIOASSORT.

In the paper III **"Second generation ethanol production from Brewers' Spent Grain"** the alkaline-acid pretreated Brewers' Spent Grain (BSG), after enzymatic hydrolysis with commercial enzymes, was evaluated as fermentation medium for ethanol production by the strain *Saccharomyces cerevisiae* NRRL YB 2293 selected as the best producer among five ethanologenic microorganims belonging to the strain collection of DEBB University, where these activities were performed during my stage

in the frame of the BIOASSORT Project. In particular, a comparison between the yields of lactic acid produced by the ethanologenic strain from BSG with or without any supplementation of yeast extract was carried out.

4.2 Results

4.2.1 Second generation ethanol production from Brewers' Spent Grain (Paper III)

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Article

Second Generation Ethanol Production from Brewers' Spent Grain

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Abstract: Ethanol production from lignocellulosic biomasses raises a global interest because it represents a good alternative to petroleum-derived energies and reduces the food *versus* fuel conflict generated by first generation ethanol. In this study, alkaline-acid pretreated brewers' spent grain (BSG) was evaluated for ethanol production after enzymatic hydrolysis with commercial enzymes. The obtained hydrolysate containing a glucose concentration of 75 g/L was adopted, after dilution up to 50 g/L, for fermentation by the strain *Saccharomyces cerevisiae* NRRL YB 2293 selected as the best producer among five ethanologenic microorganims. When the hydrolysate was supplemented with yeast extract, 12.79 g/L of ethanol, corresponding to 0.28 g of ethanol per grams of glucose consumed (55% efficiency), was obtained within 24 h, while in the non-supplemented hydrolysate, a similar concentration was reached within 48 h. The volumetric productivity increased from 0.25 g/L·h in the un-supplemented hydrolysate to 0.53 g/L h in the yeast extract supplemented hydrolysate. In conclusion, the strain *S. cerevisiae* NRRL YB 2293 was shown able to produce ethanol from BSG. Although an equal amount of ethanol was reached in both BSG hydrolysate media, the nitrogen source supplementation reduced the ethanol fermentation time and promoted glucose uptake and cell growth.

Keywords: brewers' spent grain; lignocellulosic conversion; second generation bioethanol; enzymatic saccharification; ethanologenic microorganisms

1. Introduction

The depletion of fossil feedstock and climate changes, such as global warming, due to green house gas emissions (GHGs), have caused increasing interest in alternative renewable sources of energy, pushing the worldwide trend to produce and use bio-based products and biofuels in substitution of the fossil-based ones [1].

The most abundant renewable resource produced all around the world is represented by lignocellulosic biomasses [2], which include agricultural residues, food farming wastes, "green-grocer's wastes", tree pruning residues and the organic and paper fraction of urban solid wastes. A global interest in using the lignocellulosic residues and waste as source of added value bioproducts is rising due to their renewability, low cost, abundance, and non-competitiveness with food [3], boosting the development of the biorefinery concept and advancing sustainable waste management [4].

Among the lignocellulosic residues, a great interest has been focused on brewers' spent grain (BSG). BSG is a solid residue of breweries consisting of exhausted grain husks, obtained after mashing and lautering. Its composition changes based on the operative conditions adopted during harvest, malting and mashing time [5].

Until now, BSG was employed as animal feed or deposited in landfill. However, recent studies revealed its potential application for the production of a wide range of high added value bio-products, such as organic acids, biogas, bioethanol, biopolymers and molecules for the food and pharmaceutical industries [6,7]. The feasibility of these production processes should be evaluated on the basis of local availability of BSG, its current utilization for animal feed production and the environmental and economical advantages that could be gained by changing its use to generate higher added value products. Second generation ethanol, produced from non-food renewable lignocellulosic resources, has been recognized as a good alternative to petroleum-derived transportation fuels, with several known advantages, such as high octane number, low cetane number and high heat of vaporization [8]. Furthermore, it reduces the food *versus* fuel conflict created by first generation ethanol, mainly produced in USA and Brazil by using corn and sugar cane, respectively, as a feedstock [9]. The complex structure of lignocellulosic biomasses requires a pretreatment step to remove the recalcitrant lignin, followed by the enzymatic saccharification prior to sugar fermentation into ethanol.

Several techniques, such as physical treatment, chemical (alkaline and/or acid) treatment, biological treatment, physicochemical treatment, *i.e.*, steam explosion, liquid hot water (LHW), ammonia fiber expansion (AFEX), supercritical fluid (SCF) treatment and thermochemical treatment have been explored for removing lignin [10]. Once lignin has been removed, saccharification of the free accessible (hemi)cellulose portions of the biomass is carried out to obtain a hydrolysate rich in fermentable monosaccharides [11].

In this manuscript, the screening of five ethanologenic microorganisms for their ethanol production ability was performed. The most productive strain was then tested for its ability to grow and produce

ethanol using the sugar mixture obtained by enzymatic hydrolysis of chemically pretreated BSG. Compared to the few other works so far reported on ethanol production from BSG, this study allowed achieving a higher or similar ethanol yield and a higher productivity.

2. Results and Discussion

2.1. Screening of Ethanologenic Microorganisms for Ethanol Production in a Synthetic Medium

The strains *Saccharomyces cerevisiae* NRRL Y 12,908, *S. cerevisiae* NRRL YB 2293, *S. cerevisiae* NRRL Y 11,878, *S. cerevisiae* NRRL Y 2034 and *Zigosaccharomyces rouxi* NRRL Y 2547 were investigated for their ethanol production ability in a synthetic growth medium containing 40 g/L glucose. Initial glucose was completely consumed by the five strains after 72 h of fermentation at 30 °C, while the final ethanol concentration varied from a minimum value of 9.01 ± 0.05 g/L to a maximum one of 10.82 ± 0.03 g/L (Table 1). Among the investigated strains, *S. cerevisiae* NRRL YB 2293 showed the highest fermentation yield, with a maximum value of ethanol production of 10.82 ± 0.03 g/L after 72 h and a productivity of 0.15 g/L h (Table 1). It gave an ethanol yield of 0.27 g/g, equivalent to 53% of the theoretical ethanol yield by *S. cerevisiae*.

Table 1. Glucose and ethanol concentration after 72 h of fermentation in synthetic medium of the yeast species strains. Data represent the mean of two independent experiments.

Strains	Glucose (g/L)	Ethanol (g/L)
	40.0	0.0
Saccharomyces cerevisiae NRRL Y 12,908	0.99	10.52 ± 0.02
Saccharomyces cerevisiae NRRL YB 2293	0.0	10.82 ± 0.03
Saccharomyces cerevisiae NRRL Y 11,878	0.0	9.25 ± 0.05
Saccharomyces cerevisiae NRRL Y 2034	0.0	9.56 ± 0.03
Zigosaccharomyces rouxi NRRL Y 2547	0.0	9.01 ± 0.05

2.2. Chemical Pretreatment of BSG and Saccharification of the Pretreated BSG by a Cocktail of Commercial Enzymes

BSG, as well as other lignocellulosic wastes, represents a renewable source of fermentable sugars that can be employed as a feedstock for biofuels and chemicals production.

In this study, the BSG was chemically pretreated and saccharified with a cocktail of commercial enzymes.

A chemical pretreatment of BSG was carried out following the operative conditions reported by Mussatto *et al.* [12]. The analyses of the macromolecular composition of BSG before and after the pretreatment are reported in Table 2. The untreated BSG consisted of about 14.42% (*w*/*w*) cellulose, 34.21% (*w*/*w*) hemicelluloses, 3.93% (*w*/*w*) lignin and 47.43% (*w*/*w*) other materials (ash, protein and extractives). The chemical composition of BSG varies according to barley variety, harvest time, malting, and mashing conditions [13]. It is worth noting that Murdock *et al.* [14] reported a BSG with a similar lignin and hemicellulose content of 29.9% and 3.8% (*w*/*w*), respectively. Moreover, the high content of other materials, such as proteins and fibers, detected in our study is typical for BSGs, in which those materials account for 20% to 70% of total composition [7].

Table 2. Chemical Composition (% *w*/*w*) of untreated, pretreated BSG.

Component	Untreated BSG	Pretreated BSG
Cellulose	14.42	86.49
Hemicellulose	34.21	3.87
Lignin	3.93	2.31
Others (ash, protein and extractives)	47.43	7.33

A first treatment with sulfuric acid was performed to solubilize the hemicellulosic fraction and increase the diffusion of sodium hydroxide into the lignocellulosic structure, thus enhancing soda pulping. The alkaline pretreatment, performed to liberate cellulose fibers from lignin [15], is not totally selective for lignin, and the carbohydrates, including cellulose, can also be degraded [16]. The immediate cooling of the reaction medium in an ice bath allowed reducing the sugar loss and obtaining a solid residue with high cellulose content [17].

After the pretreatment, a total biomass recovery yield of 7.89% (*w*/*w*) was obtained. Considering an initial amount of 100 g of dried BSG, the hemicellulose was the main removed fraction, with a recovery of only 3.87 g from the 34.21 g present in the initial biomass, while 2.31 g of lignin were recovered from the 3.93 g treated. The pretreatment also promoted a high hydrolysis of the other compounds (ash, protein and extractives) with a recovery of 7.33 g from the 27 g treated.

The composition of BSG after the complete pretreatment consisted of about 86.49% (*w*/*w*) cellulose, corresponding to the main fraction, 3.87% (*w*/*w*) hemicelluloses, 2.31% (*w*/*w*) lignin and 7.33% (*w*/*w*) other materials (Table 2).

The cellulose pulp obtained after pretreatment was saccharified with a cocktail of commercial enzymes. At the end of the reaction, a hydrolysate with a final glucose concentration equal to 75 g/L, a value higher than those obtained in the other few studies so far reported on the use of hydrolysates from agricultural residues as fermentation media for ethanol production (Table 3). In particular, the value of 97% efficiency for cellulose conversion into glucose attained in the saccharification step was higher than the efficiency of 72% obtained by Mussatto *et al.* on a similarly pretreated BSG [12]. The glucose yield was calculated according to the following equation:

$$\text{Glucose yield (\%)} = (\text{Glucose} \times 0.9 \times 100)/(\text{cellulose content in the substrate}) \quad (1)$$

Table 3. Ethanol production from BSG hydrolysates.

Waste Type	Waste Pretreatment	Enzymatic Hydrolysis	Yield of Glucose after Enzymatic Hydrolysis	Microorganism Used in Fermentation Step	Ethanol Production	References
BSG	1.25% (*v*/*v*) H_2SO_4 in a ratio of 1:8 (*w*/*w*) at 120 °C for 17 min following by treatement with 2% (*v*/*v*) NaOH in a 1:20 (*w*/*w*) ratio at 120 °C for 90 min	2.24% (*v*/*v*) cellulase (Novozymes) and 1% (*v*/*v*) β-glucosidase (Novozymes), using 8% (*w*/*v*) * substrate at 45 °C and 120 rpm for 72 h	75 g/L glucose corresponding to 97% efficiency of cellulose conversion into glucose	*Saccharomyces cerevisiae* NRRL YB 2293	12.0 g/L ethanol, corresponding to 0.26 g ethanol/g substrate in BSG hydrolysate without adding any nutrients supplementation and 12.79 g/L ethanol, corresponding to 0.28 g ethanol/g in the yeast extract-supplemented BSG	This study
BSG	20% (*w*/*v*) BSG, pretreated with 0.16 N HNO_3 at 121 °C for 15 min, partially neutralized to pH 5–6 with NaOH	Novozymes Biomass sample kit (cocktail of 42.0 (U/g) ** cellulase, 1.5 (U/g) β-glucosidase, 3.0 (U/g) hemicellulase and 2.5 (U/g) xylanase) for 18 h at 50 °C, 130 rpm	27 g/L glucose, 16.7 g/L xylose and 11.9 g/L arabinose	*Pichia stipitis* NCYC 1540 and	8.3 g/L corresponding to ethanol conversion yields of 0.32 g ethanol/g substrate	[18]
BSG	12.5% (*w*/*v*) BSG, pretreated with 2.5 M NaOH at 121 °C for 30 min, neutralized to pH 5–6 with H_2SO_4	17.0 (U/g) of xylanase and 3.21 (U/g) of endoglucanase from *Fusarium oxysporum* for 24 h at 30 °C, 1400 rpm	52 g/L glucose	*Fusarium oxysporum* F3	Ethanol conversion yields of 0.065 g ethanol/g substrate	[19]
BSG	20% (*w*/*v*) BSG, pretreated with 0.16 N HNO_3 at 121 °C for 15 min, partially neutralized to pH 5–6 with NaOH	Novozymes Biomass sample kit (cocktail of 42.0 (U/g) cellulase, 1.5 (U/g) β-glucosidase, 3.0 (U/g) hemicellulase and 2.5 (U/g) xylanase) for 18 h at 50 °C, 130 rpm	27 g/L glucose, 16.7 g/L xylose and 11.9 g/L arabinose	*Pichia stipitis* NCYC 1540	14.8 g/L ethanol	[20]
BSG	25% (*w*/*v*) BSG, pretreated with 5% (*w*/*v*) NaOH at 50 °C for 12 h	51 Filter Paper Uunit (FPU)/g Cellic®CTec2 (Novozymes) for 24 h	41.7 g/L glucose and 14.6 xylose	*Saccharomyces cerevisiae* strain NCYC479	17.3 g/L ethanol, corresponding to *ca.* 81% of theoretical ethanol yield	[21]
BSG	BSG treated with 7% (*w*/*v*) H_2SO_4 at 96 °C for 3 h	Enzymatic cocktail produced by *Fusarium oxysporum* F3 under submerged conditions	-	*Fusarium oxysporum* F3	109 g/Kg of substrate, corresponding to 60% of theoretical ethanol yield	[22]

Notes: * (*w*/*v*): weight/volume; ** (U/g): enzymatic unit per gram of substrate.

This result could be explained by a different composition of the BSG used in the two studies. Before and after the pretreatment, the BSG employed in this work and in that of Mussatto *et al.* had a similar cellulose and hemicellulose composition [12], but they differed in the content of lignin, which was higher in the material investigated by Mussatto *et al.* [12]. It is possible that the lignin content of 8.2% (*w*/*w*) for the BSG used by Mussatto *et al.*, compared to a value of 2.31% (*w*/*w*) for the BSG employed in this work, had a negative effect (i) during the alkaline pulping process, decreasing the lignin loss and (ii) during the saccharification, reducing the efficiency of cellulose conversion into glucose [12]. Moreover, it was reported that different pretreatment methods could influence the saccharification yield. The decrease of the BSG particle size to the micron level allowed improving the carbohydrate solubilization yield from 23% up to 45% by using a multi-enzyme mixture, as reported by Niemi *et al.* [23]. Xiros *et al.* [24] demonstrated that the yield of enzymatic hydrolysis of alkali pre-treated BSG, through the enzyme extract from *Neurospora crassa*, increased by about 50% in comparison with the non-pretreated material, achieving about 50% and 60% yield of pentose and glucose, respectively.

2.3. Ethanol Production Using BSG Hydrolysate as Fermentation Medium

The strain *S. cerevisiae* NRRL YB 2293, previously selected among the investigated ethanologenic strains, was analyzed for its ability to grow on the sugar mixture obtained by enzymatic hydrolysis of chemically pretreated BSG. The BSG hydrolysate was adopted as growth medium with or without 1.25% yeast extract supplementation. The medium was diluted to a glucose concentration of 50 g/L and the pH was adjusted to 6.0.

The strain was able to grow in both media, showing that the BSG hydrolysate is a potential fermentation medium for ethanol production. The ethanol yield per substrate consumed ($Y_{P/S}$) was almost the same in the two different media: 0.26 g/g (51% efficiency) and 0.28 g/g (55% efficiency) when the BSG hydrolysate was used without adding any additional element and in the yeast extract-supplemented formulation, respectively (Table 4). However, the cell biomass increased when the yeast extract was added to the BSG hydrolysate. Hence, the ethanol yield per cell mass ($Y_{P/X}$) obtained in the no supplemented medium was much higher than that reached in the yeast extract supplemented BSG hydrolysate (2.8 g/g *versus* 1.7 g/g, respectively) (Table 4).

Table 4. Fermentative parameters of ethanol production by *Saccharomyces cerevisiae* NRRL YB 2293 from BSG hydrolysate and BSG hydrolysate + yeast extract.

Medium composition	Glucose Consumption (g/L)	Ethanol (g/L)	$Y_{P/S}$ (g/g) [a]	$Y_{P/X}$ (g/g) [b]	Q_P (g/L h) [c]	η (%) [d]
BSG hydrolysate	45.0	12.0	0.26	2.8	0.25	51
BSG hydrolysate + yeast extract	45.0	12.79	0.28	1.7	0.53	55

Notes: [a] g-ethanol produced/g-glucose consumed; [b] g-ethanol/g-dry cell weight; [c] (g/L) ethanol/(h) fermentation time; [d] $Y_{P/S}$/maximum theoretical value (0.51 g/g).

Even though all the glucose was consumed by the cells in both media, and the same ethanol concentration was reached, it is worth noting that the yeast extract supplementation allowed increasing the volumetric productivity: the cells produced 12.79 ± 1.2 g/L of ethanol, completed the glucose

uptake and reached the highest cell dry biomass (7.01 ± 1.3 g/L) within 24 h, while in the BSG hydrolysate without any nutrient supplementation, all the glucose was used by the cells, achieving almost the same ethanol concentration of 12.0 ± 1.2 g/L, and reaching the highest cell dry biomass (4.25 ± 1.1 g/L) only after 48 h (Figure 1) (Table 4). In fact, the volumetric productivity in BSG hydrolysate was 0.25 g/L·h, two-fold lower than the value reached in the yeast supplemented BSG hydrolysate (0.53 g/L·h) and the ethanol yield per cell mass ($Y_{P/X}$) obtained in the no supplemented medium was 2.7 g/g, much higher than that reached in the yeast extract supplemented medium (1.7 g/g) (Table 4).

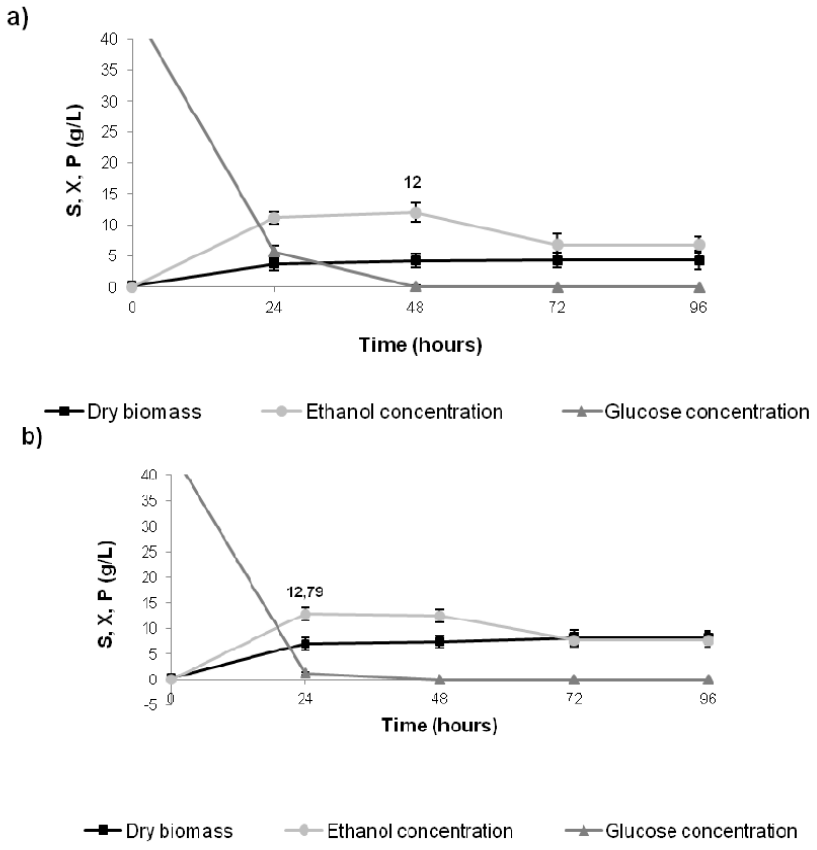

Figure 1. Fermentation of glucose to ethanol by *Saccharomyces cerevisiae* NRRL YB 2293 in (**a**) BSG hydrolysate and (**b**) BSG hydrolysate + yeast extract; glucose concentration (*S*, Δ), dry biomass concentration (*X*, □) and ethanol concentration (*P*, ○). All values are mean of two independent experiments.

These results showed clearly that the nitrogen source supplementation allowed a better growth of the cells, which completed the glucose uptake and reached the fermentation ethanol rate in 24 h instead of 48 h, as reported by Kolothumannil and Ingledew [25]. They demonstrated that the yeast extract supplementation to the wheat mashes hydrolysate reduced the ethanol fermentation time and promoted the glucose uptake and cell growth: an ethanol yield of 17.1% (*v*/*v*) was obtained within three days when yeast extract was added, while in the absence of nutrient supplementation, a final ethanol yield of 16.9% (*v*/*v*) was still achieved but after eight days.

Few works on BSG conversion into ethanol are so far reported and the results of these studies are summarized in Table 3. The different pretreatment methods, enzymatic cocktails for the saccharification step, ethanologenic microorganisms and growth conditions employed in these works influenced the ethanol yield.

Recently, Wilkinson *et al.* performed a study targeted at establishing the best BSG treatment, among the NaOH- or alkaline peroxide-based methods [21], to maximize the release of sugars during enzymatic hydrolysis. Pre-treatment with 5% NaOH at 50 °C for 12 h at 25% (*w*/*v*) solids, followed by a saccharification with an excess commercial enzyme resulted in the best pretreatment method for the BSG. The operative conditions of each step were completely different from our work, influencing the fermentation rate, but not the final ethanol concentration (Table 3). As a matter of fact, in 10 days of fermentation, they obtained almost the same ethanol yield reached in only one day by our strain *S. cerevisiae* NRRL YB 2293 (17.3 g/L *vs.* 12.79 g/L).

An ethanol yield production of 0.065 g/g from dry BSG, very low in comparison to 0.28 g/g obtained by *S. cerevisiae* NRRL YB 2293, was reported by Xiros *et al.* [19] (Table 3). However, the authors adopted very different operative conditions to produce ethanol by employing the mesophilic fungus *Fusarium oxysporum* and coupling alkali BSG pretreatment with solid-state (for enzymes production by the fungus) and submerged fermentation (for ethanol production).

The concentration of ethanol obtained from pretreated BSG by the strain *Pichia stipitis* NCYC 1540 was lower than that reached by *S. cerevisiae* NRRL YB 2293 (8.3 g/L *vs.* 12.79 g/L, respectively) (Table 3), although the ethanol yield is almost the same (0.32 g/g *vs.* 0.26 g/g, respectively) [18]. Even after optimization (effect of pH, other toxic inhibitors or lack of nutrients), performed by Yohannan *et al.* [20], the same strain gave a comparable ethanol production (~14.8 g/L) (Table 3) to that obtained in this work (12.79 g/L). Recently, Xiros and Christakopoulos [22] obtained a high ethanol yield from BSG by using the fungus *Fusarium oxysporum*. The (hemi)cellulolytic fungal enzymes, produced under submerged fermentation on BSG, were used for the saccharification of alkali-pretreated BSG; the obtained hydrolysate was converted into ethanol by the fungus in a consecutive submerged fermentation. The optimized-process allowed obtaining an ethanol yield of 109 g/Kg dry BSG, corresponding to 60% of the theoretical ethanol yield.

In conclusion, compared to the few other works so far reported on ethanol production from BSG, this study achieved a higher or similar ethanol yield and a higher productivity.

3. Experimental Section

3.1. Microorganisms and Cultivation Conditions for Screening in Synthetic Medium

The microorganisms investigated in this study include the strains *Saccharomyces cerevisiae* NRRLY 12,908, *S. cerevisiae* NRRL YB 2293, *S. cerevisiae* NRRL Y 11,878, *S. cerevisiae* NRRL Y 2034 and *Zigosaccharomyces rouxi* NRRL Y 2547, all belonging to the strain collection of the Bioprocess and Biotechnology Division of the Department of "Engenharia de Bioprocessos e Biotecnologia" (University Federal do Paraná, Brasil). Cell cultures were maintained in 10 g/L glycerol at −20 °C. One milliliter of stock culture cells was transferred to 25 mL test tubes containing 10 mL sterile YM broth medium with the following composition: 10 g/L glucose, 5 g/L peptone,

3 g/L malt extract and 3 g/L yeast extract. After incubation at 30 °C and 120 rpm for 24 h, 5 mL of pre-inoculum were inoculated in 125 mL Erlnemeyer flask containing 50 mL of synthetic fermentation broth with the following composition: 40 g/L glucose, 5 g/L peptone, 3 g/L malt extract and 3 g/L yeast extract. The flasks were incubated at 30 °C and 120 rpm for 72 h. Samples collected at time zero and after 72 h were subjected to analytical tests.

3.2. Chemical Pretreatment of BSG and Enzymatic Hydrolysis of Pretreated Material

The BSG, kindly provided by the brewery Bier Hoff Curitiba-PR (Brazil), was pretreated by using 1.25% (*v*/*v*) H_2SO_4 in a ratio of 1:8 (*w*/*w*) at 120 °C for 17 min [15]. After washing with water until neutral pH and drying over night at 50 ± 5 °C, the solid residue was treated with 2% (*v*/*v*) NaOH in a 1:20 (*w*/*w*) ratio at 120 °C for 90 min [26]. The residual cellulose pulp was washed and dried over night at 50 ± 5 °C. The chemical composition of the untreated and treated BSG was determined by the Animal Nutritional Laboratory of the Federal University of Paraná, using the method of Van Soest [27,28]. Saccharification was performed with 2.24% (*v*/*v*) cellulase (Novozymes) and 1% (*v*/*v*) β-glucosidase (Novozymes), using 8% (*w*/*v*) substrate at 45 °C and 120 rpm for 72 h. The hydrolysate was collected after centrifugation.

3.3. Inoculum and Fermentation Conditions for Cultivation in BSG Hydrolysate

The strain selected as the best producer of ethanol in the synthetic fermentation broth, *S. cerevisiae* NRRL YB 2293, was pre-inoculated in 25 mL tubes containing 10 mL of YM broth. After incubation at 120 rpm for 24 h at 30 °C, 1 mL of this culture was transferred to a new tube with 10 mL of YM broth, and incubated at the same conditions. After 24 h, cells were collected by centrifugation at 10,000 rpm for 15 min and washing three times in sterilized water. Ten percent (*v*/*v*) of this suspension was added to 10 mL of BSG hydrolysate with or without the addition of 1.25% (*w*/*v*) yeast extract. The fermentation media were adjusted to a pH of 6.0 by adding 5 M NaOH and diluting up to 50 g/L of glucose concentration. Incubation was performed at 30 °C and 120 rpm for 96 h. Samples for High-Performance Liquid Chromatography analyses were collected every 24 h and all of the assays were performed in duplicate.

3.4. Analytical Methods

Samples taken during the fermentation were centrifuged at 13,000 rpm for 15 min. Glucose and ethanol concentrations were measured by High-Performance Liquid Chromatography (HPLC). The analyses were performed in a Shimadzu LC-10-AD equipped with a C-RSA Integrator Chromatopac Chromatography Unit (Shimadzu, Kyoto, Japan) set to 210 nm and (300 mm × 7.8 mm) column Aminex HPX-87-4 (Bio-Rad Labs, Richmond, CA, USA), and a refractive index detector. Before the injection into the chromatograph, samples were diluted with deionized water and filtered through 0.2 µm cellulose acetate filters (Sartorius Biolab Products, Goettingen, Germany). The conditions of chromatography used were: a column temperature of 60 °C, a mobile phase of 5 mM sulfuric acid at a flow rate of 0.6 mL/min, and an injection volume of 50 µL. Calibration curves obtained using standard solutions were utilized to calculate the glucose and ethanol concentration. Cell optical density (OD)

was spectrophotometrically measured at 600 nm by using the spectrophotometer SP-2000 (Shanghai Spectrum Instruments Co., Shanghai, China). The OD values were correlated with the cell concentration (g/L) by means of a standard calibration curve previously established. One unit of optical density at 600 nm corresponded to approximately ~0.75 g dry cell weight/L.

3.5. Analysis of Fermentative Parameters

The ethanol yield per substrate consumed ($Y_{P/S}$ g/g), the volumetric productivity (Q_p g/L h), the ethanol yield per cell mass ($Y_{P/X}$ g/g), and the efficiency (η, %) were evaluated for each sample collected during the fermentations.

4. Conclusions

Among the investigated strains, *Saccharomyces cerevisiae* NRRL YB 2293, belonging to the collection of the Bioprocess and Biotechnology Division of the Department of "Engenharia de Bioprocessos e Biotecnologia" (University Federal do Paraná, Brasil), was selected for its higher ability to produce ethanol in a synthetic medium. It was then applied for conversion of BSG hydrolysate into ethanol.

BSG was subjected to a chemical pretreatment by acid-alkali method and saccharified with a cocktail of commercial enzymes, obtaining 97% efficiency of cellulose conversion into glucose.

The strain *S. cerevisiae* NRRL YB 2293 was able to grow and to produce ethanol on the BSG hydrolysate with and without adding yeast extract. The ethanol yield per substrate consumed ($Y_{P/S}$) was almost the same in both media: 0.26 g/g (51% efficiency) and 0.28 g/g (55% efficiency) when the BSG hydrolysate was used without adding any additional element and in the yeast extract-supplemented BSG, respectively.

Yeast extract supplementation promoted cell growth, glucose uptake and ethanol fermentation at a higher rate. When the BSG was supplemented with yeast extract, the cells produced 12.79 g/L ethanol, completed the glucose uptake and reached the highest cell dry biomass (7.01 g/L) within 24 h, while in the BSG hydrolysate without any nutrient supplementation, all the glucose was consumed by the cells, achieving almost the same ethanol concentration of 12.0 g/L and reaching the highest cell dry biomass (4.25 g/L), within 48 h.

The volumetric productivity in the unsupplemented BSG was 0.25 g/L h, twofold lower than the value reached in the yeast extract supplemented BSG (0.53 g/L h) and the ethanol yield per cell mass ($Y_{P/X}$) obtained in the non-supplemented medium was 2.7 g/g, much higher than that reached in the yeast extract supplemented medium (1.7 g/g).

The application of the strain *S. cerevisiae* NRRL YB 2293 on BSG hydrolysate allowed obtaining a higher or equal ethanol yield compared to the few other works so far reported.

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Author Contributions

Rossana Liguori carried out the experiments of screening of the ethanologenic microorganisms; chemical pretreatment of BSG and enzymatic saccharification of the solide residue remaining after pretreatment; and test of the most productive strain in ethanol production from BSG hydrolysate; Carlos Ricardo Soccol contributed to conceiving the study and participated in its design and coordination for the part of BSG pretreatment and saccharification; Luciana Porto de Souza Vandenberghe contributed to conceiving the study and participated in its design and coordination for the part of BSG conversion and analyzed the data; Adenise Lorenci Woiciechowski carried out the HPLC analyses; Vincenza Faraco contributed to conceiving the study and participated in its design and coordination for the part of screening of microorganisms and conversion of BSG hydrolysate and drafted the manuscript; All authors read and approved the final manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Amore, A.; Giacobbe, S.; Liguori, R.; Faraco, V. The second generation ethanol production. *Rend. Accad. Naz. Sci. XL Mem. Sci. Fis. Naur.* **2014**, *XXXVII*, 113–136.
2. Prasad, S.; Singh, A.; Joshi, H.C. Ethanol as an alternative fuel from agricultural, industrial and urban residues. *Conserv. Recycl.* **2007**, *50*, 1–39.
3. Liguori, R.; Amore, A.; Faraco, V. Waste valorization by biotechnological conversion into added value products. *Appl. Microbiol. Biotechnol.* **2013**, *97*, 6129–6147.
4. Dusselier, M.; Mascal, M.; Sels, B.F. Top chemical opportunities from carbohydrate biomass: A chemist's view of the biorefinery. *Top. Curr. Chem.* **2014**, *353*, 1–40.
5. Santos, M.; Jiménez, J.J.; Bartolome, B.; Gómez-Cordovés, C.; del Nozal, M.J. Variability of brewers' spent grain within a brewery. *Food Chem.* **2003**, *80*, 17–21.
6. Mussatto, S.I. Brewer's spent grain: A valuable feedstock for industrial applications. *J. Sci. Food Agric.* **2014**, *94*, 1264–1275.
7. Mussatto, S.I.; Dragone, G.; Roberto, I.C. Brewers' spent grain: Generation, characteristics and potential applications. *J. Cereal Sci.* **2006**, *43*, 1–14.
8. Balat, M. Global trends on the processing bio-fuel. *Energy Explor. Exploit.* **2007**, *25*, 195–218.
9. Pimentel, D.; Marklein, A.; Toth, M.A.; Karpoff, M.; Paul, G.S.; McCormack, R.; Kyriazis, J.; Krueger, T. Biofuel impacts on world food supply: Use of fossil fuel, land and water resources. *Energies* **2008**, *1*, 41–78.
10. Chundawat, S.; Beckham, G.; Himmel, M.; Dale, B. Deconstruction of lignocellulosic biomass to fuels and chemicals. *Annu. Rev. Chem. Biomol. Eng.* **2011**, *2*, 121–145.
11. Sun, Y.; Cheng, J. Hydrolysis of lignocellulosic materials for ethanol production: A review. *Bioresour. Technol.* **2002**, *83*, 1–11.
12. Mussatto, S.I.; Fernandes, M.; Dragone, G.; Mancilha, I.M.; Roberto, I.C. Brewers' spent grain as raw material for lactic acid production by *Lactobacillus delbrueckii*. *Biotechnol. Lett.* **2007**, *29*, 1973–1976.

13. Muthusamy, N. Chemical composition of brewers spent grain—A review. *Int. J. Sci.* **2014**, *3*, 2109–2112.
14. Murdock, F.R.; Hodgson, A.S.; Riley, R.E., Jr. Nutritive value of brewers grains for lactating dairy cows. *J. Dairy Sci.* **1981**, *64*, 1826–1832.
15. Mussatto, S.I.; Roberto, I.C. Acid hydrolysis and fermentation of brewers' spent grain to produce xylitol. *J. Sci. Food Agric.* **2005**, *85*, 2453–2460.
16. Clayton, D.; Easty, D.; Einspahr, D.; Lonsky, W.; Malcolm, E.; McDonough, T.; Schroeder, L.; Thompson, N. In *Pulp and Paper Manufacture*, 3rd ed.; Grace, T.M., Malcolm, E.W., Eds.; The Joint Textbook Committee of the Paper Industry: Montreal, QC, Canada, 1989; Volume 5, pp. 1–128.
17. Knill, C.J.; Kennedy, J.F. Degradation of cellulose under alkaline conditions. *Carbohydr. Polym.* **2003**, *51*, 281–300.
18. White, J.S.; Yohannan, B.K.; Walker, G.M. Bioconversion of brewer's spent grains to bioethanol. *FEMS Yeast Res.* **2008**, *8*, 1175–1184.
19. Xiros, C.; Topakas, E.; Katapodis, P.; Christakopoulos, P. Evaluation of *Fusarium oxysporum* as an enzyme factory for the hydrolysis of brewer's spent grain with improbe biodegradability for ethanol production. *Ind. Crop. Prod.* **2008**, *28*, 213–224.
20. Yohannan, B.K.; White, J.S.; Bennett, J.; Walker, G.M. Distiller's spent grains: A substrate for bioethanol? In *Distilled Spirits: New Horizons: Energy, Environment and Enlightenment*, Proceedings of the 3rd Worldwide Conference on Distilled Spirits, Edinburgh, Scotland, UK, 7–10 September 2008.
21. Wilkinson, S.; Smart, K.A.; Cook, D.J. Optimisation of alkaline reagent based chemical pre-treatment of Brewers spent grains for bioethanol production. *Ind. Crop. Prod.* **2014**, *62*, 219–227.
22. Xiros, C.; Christakopoulos, P. Enhanced ethanol production from brewer's spent grain by a *Fusarium oxysporum* consolidated system. *Biotechnol. Biofuels* **2009**, *2*, doi:10.1186/1754-6834-2-4.
23. Niemi, P.; Faulds, C.B.; Sibakov, J.; Holopainen, U.; Poutanen, K.; Buchert, J. Effect of a milling pre-treatment on the enzymatic hydrolysis of carbohydrates in brewer's spent grain. *Bioresour. Technol.* **2012**, *116*, 155–160.
24. Xiros, C.; Topakas, E.; Katapodis, P.; Christakopoulos, P. Hydrolysis and fermentation of brewer's spent grain by *Neurospora crassa*. *Bioresour. Technol.* **2008**, *99*, 5427–5435.
25. Kolothumannil, C.T.; Ingledew, W.M. Fuel alcohol production: Effects of free amino nitrogen on fermentation of very-high-gravity wheat mashes. *Appl. Environ. Microbiol.* **1990**, *56*, 2046–2050.
26. Mussatto, S.I.; Dragone, G.; Rocha, G.J.M.; Roberto, I.C. Optimum operating conditions for brewers' spent grain soda pulping. *Carbohydr. Polym.* **2006**, *64*, 22–28.
27. Van Soest, P.J.; Wine, R.H. Use of detergents in the analysis of fibrous feeds. IV Determination of plant cell-wall constituents. *Assoc. Off. Agric. Chem. J.* **1967**, *50*, 50–55.
28. Van Soest, P.J. Development of a comprehensive system of feed analysis and its application to forage. *J. Anim. Sci.* **1967**, *26*, 119–120.

Chapter V

Concluding remarks

This PhD project was aimed at the development of biosystems and biocatalysts, (microbes and/or enzymes), involved in lignocellulosic biomass conversion into valuable bio-products, such as sugars, ethanol and lactic acid.

As a source of new biosystems (lignocellulolytic microorganisms) and biocatalysts (ligninolytic, cellulolytic and xylanolytic enzymes), 32 fungal strains of the "Strain Collection of the Bioprocess and Biotechnology Division" from the Department of "Engenharia de Bioprocessos e Biotecnologia", University Federal do Paraná (DEBB) (Curitiba-PR, Brasil) were analyzed for their ability to produce the enzymes of interest by screening them on solid and liquid media. This led to the selection of the ligninolytic strain *Pleurotus sajor-caju* INRA 3501 and the (hemi)cellulolytic strain *Aspergillus niger* NRRL 3312. The low-cost production of ligninolytic, cellulolytic and xylanolytic enzymes has been optimized by setting up solid state fermentation on lignocellulosic waste Brewers' spent grain (BSG), reaching a maximum value of cellulase, xylanase and laccase activity of 118.04 U g^{-1} of dry substrate (gds), 1315.15 U gds^{-1} and 157.36 U gds^{-1}, respectively. By proteomic analyses peptides belonging to the enzymes responsible for the cellulase and xylanase activities from *A. niger* NRRL 3312 and for laccase activity from *P. sajor-caju* INRA 3501 were identify. The results supported the use of lignocellulosic waste as a low-cost source for industrial enzymes production; moreover, the new enzymes will be explored for their potential role in lignocellulosic biomass delignification and saccharification, thus evaluating their potential contribution to improve cost efficiency of high added value bioproducts production.

The inductive effect of microcrystalline cellulose on (hemi)cellulolytic enzymes production by *Pleurotus ostreatus* was proved, reaching a maximum values of 3.19 U/mL and 51.32 U/mL for cellulases and xylanases, respectively, after 9 days of fermentation. Thus, the enzymatic mixture from *P. ostreatus* was partially characterized and applied to the hydrolysis of the lignocellulosic biomass *Arundo donax*. The enzymes showed stability in a wide range of pH, with an optimum values of 4.0 and 5.3 for cellulase and xylanase activity, respectively; both enzymes have an optimum of temperature at 50 °C, value generally used during the saccharification of lignocellulose. Regarding the pH stability, the cellulase from *P. ostreatus* showed a half-life of 7 hours at both 30 °C and 40 °C, while the xylanase showed a half-life of 2 days at 30 °C and 7 hours at 40 °C. Then, the enzymatic cocktail from *P. ostreatus* was tested in the statistical analysis of *Arundo donax* saccharification, with the aim of optimizing the process through the identification of the most significant parameters influencing the sugars release. By the Plackett-Burman screening design, the parameters temperature, pH and time were identified as the most significant variables for the process and thus, their combined effect were investigated by a 3^3 factorial experimental design. The maximum value of 480.10 mg of sugars gds^{-1} was obtained at 45 °C, pH 3.5, and 96 hours of incubation; moreover, a relevant positive impact on the bioconversion process was exercised by the synergic effect of temperature and time. Based on the results, the (hemi)cellulolytic cocktail produced by *P. ostreatus* represent a good candidate as biocatalyst for the bioconversion of lignocellulose in substituition to the commercial ones.

Saccharomyces cerevisiae NRRL YB 2293, belonging to the DEBB collection was selected for its higher ability to produce ethanol in a synthetic medium and was further applied for the conversion of BSG hydrolysate into ethanol. It has been shown that the strain was able to grow and produce ethanol on the BSG hydrolysate with and without added yeast extract. The ethanol yield per substrate consumed ($Y_{P/S}$) was almost the same in both media: 0.26 g/g (51 % efficiency) with the BSG

hydrolysate and 0.28 g/g (55 % efficiency) with the yeast extract supplemented BSG hydrolysate. Yeast extract supplementation promoted cell growth, glucose uptake and ethanol fermentation at a higher rate. When the BSG was supplemented with yeast extract, the cells produced 12.79 g/L ethanol, completed the glucose uptake and reached the highest cell dry biomass (7.01 g/L) within 24 h, while in the BSG hydrolysate without any nutrient supplementation, all the glucose was consumed by the cells, achieving almost the same ethanol concentration of 12.0 g/L and reaching the highest cell dry biomass (4.25 g/L), within 48 h. The volumetric productivity in the unsupplemented BSG was 0.25 g/L h, two-fold lower than the value reached in the yeast extract supplemented BSG (0.53 g/L h). The results demonstrate the feasibility of ethanol production from the BSG hydrolysate by the application of the strain *S. cerevisiae* NRRL YB 2293. Indeed a higher or equal ethanol yield compared to the few other works so far reported was obtained.

The strain *Lactobacillus acidophilus* ATCC 43121, selected for its higher lactic acid production in a synthetic medium, was analyzed for its ability to produce lactic acid from BSG hydrolysate. The results indicated that the produced hydrolysates from ammonia soaking treated BSG2 (AAS BSG2), provided by the microbrewery Maneba (Striano, Naples, Italy), and from the acid-alkaline treated BSG1 (AAT BSG1), provided by the brewery Bier Hoff Curitiba-PR (Brazil), are suitable substrates for the growth of the strain *L. acidophilus* ATCC 43121 and the production of lactic acid. Moreover, it was demonstrated that the bioconversion of glucose into lactic acid was positively affected by the presence of yeast extract. The maximum value of lactic acid production (22.16 g/L) was obtained from the AAS BSG2 hydrolysate in the presence of yeast extract. The produced levels of lactic acid were comparable or higher in comparison with those obtained from hydrolysates of other agricultural residues.

The efforts of this PhD project support the actualization of a circular economy through the employment of lignocellulosic biomass as renewable resources, that can be used again and again to generate valuable and marketable products, replacing the exhaustible fossil-based resources, within the biorefinery concept.

References

[1] International Energy Agency 2015
[2] Klass DL (1998). Biomass for Renewable Energy, Fuels, and Chemicals, Academic Press, San Diego
[3] Kajaste R (2014). Chemicals from biomass-managing greenhouse gas emissions in biorefinery production chains-a review. J Clean Prod 75: 1-10
[4] Fagnano M, Impagliazzo A, Mori M, Fiorentino N (2015). Agronomic and environmental impacts of giant reed (*Arundo donax L.*): results from a long-term field experiment in hilly areas subject to soil erosion. Bioenerg Res 8: 415-422
[5] Iqbal HMN, Kyazze G, Keshavarz T (2013) Advances in the valorization of lignocellulosic materials by biotechnology: an overview. Biores 8(2): 3157-3176
[6] Anwar Z, Gulfraz M, Irshad M (2014). Agro-industrial lignocellulosic biomass a key to unlock the future bio-energy: A brief review. J Radiat Res Appl Sci 7(2): 163-173
[7] Brodeur G, Yau E, Badal K, Collier J, Ramachandran KB, Ramakrishnan S (2011). Chemical and physicochemical pretreatment of lignocellulosic biomass: a review. Enzyme Res 24; 2011:787532. Epub 2011 May 24
[8] Chandel AK, Gonçalves BCM, Strap JL, da Silva SS (2015). Biodelignification of lignocellulose substrates: An intrinsic and sustainable pretreatment strategy for clean energy production. Crit Rev Biotechnol 35(3): 281-293
[9] Chaturvedi V, Verma P (2013). An overview of key pretreatment processes employed for bioconversion of lignocellulosic biomass into biofuels and value added products. 3 Biotech 3: 415-431
[10] Tian XF, Fang Z, Guo F (2012). Impact and prospective of fungal pre-treatment of lignocellulosic biomass for enzymatic hydrolysis. Biofuels Bioprod Bioref 6(3): 335-350
[11] Vivekanand V, Dwivedi P, Sharma A, Sabharwal N, Singh RP (2008). Enhanced delignification of mixed wood pulp by *Aspergillus fumigatus* laccase mediator system. World J Microbiol Biotechnol 24: 2799-804
[12] Erdei B, Frankó B, Galbe M, Zacchi G (2012). Separate hydrolysis and co-fermentation for improved xylose utilization in integrated ethanol production from wheat meal and wheat straw. Biotechnol Biofuels 5: 12-24
[13] Balat M (2011). Production of bioethanol from lignocellulosic materials via the biochemical pathway: A review. Energ Convers Manage 52: 858-875
[14] Demirbas MF, Balat M, Balat H (2009). Potential contribution of biomass to the sustainable energy development. Energy Convers Manage 50:1746-60
[15] Srivastava N, Rawat R, Oberoi HS, Ramteke PW (2015). A review on fuel ethanol production from lignocellulosic biomass. Int J Green Energy 12(9): 949-960
[16] Renewable Fuels Association, Ethanol Industry Outlook 2008-2014 reports. (www.ethanolrfa.org/pages/annual-industry-outlook)
[17] Pimentel D, Marklein A, Toth MA, Karpoff M, Paul GS, McCormack R, Kyriazis J, Krueger T (2008). Biofuel impacts on world food supply: use of fossil fuel, land and water resources. Energies 1: 41-78
[18] Martinez FAC, Balciunas EM, Salgado JS, González JMD, Converti A, de Souza Oliveira RP (2013). Lactic acid properties, applications and production: A review. Trend Food Sci Tech 30: 70-83
[19] Salminen S, Ouwehand A, Wright AV, Daly C (1993). Future aspects of research and product development of lactic acid bacteria. In S. Salminen, A. von Wright, & A. Ouwehand (Eds.), Lactic acid bacteria microbiological and functional aspects (pp. 429e432). New York: Marcel Dekker, Inc
[20] Gao C, Ma C, Xu P (2011). Biotechnological routes based on lactic acid production from biomass. Biotechnol Adv 29(6): 930-939
[21] San-Martín M, Pazos C, Coca J (1992). Reactive extraction of lactic acid with alamine 336 in the presence of salts and lactose. J Chem Technol Biot 54: 1-6
[22] John RP, Sukumaran R, Nampoothiri KM, Pandey A (2007). Statistical optimization of simultaneous saccharification and L(+)-lactic acid fermentation from cassava bagasse using

mixed culture of lactobacilli by response surface methodology. Biochemical Engineering Journal, 36(3), 262-267
[23] Jawad AH, Alkarkhi AFM, Jason OC, Easa AM, Norulaini NAN (2013). Production of the lactic acid from mango peel waste-factorial experiment," Journal of King Saud University-Science 25(1): 39-45
[24] McCaskey TA, Zhou SD, Britt SN, Strickland R (1994). Bioconversion of municipal solid waste to lactic acid by *Lactobacillus* species," Appl Biochem Biotechnol 45(1): 555-568
[25] Mussatto SI, Fernandes M, Mancilha IM, Roberto IC (2008). Effects of medium supplementation and pH control on lactic acid production from Brewer's spent grain. Biochemical Engineering Journal 40(3): 437-444
[26] Agler MT, Wrenn BA, Zinder SH, Angenent LT (2011). Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. Trends Biotechnol 29(2): 70-78
[27] Karp SG, Faraco V, Amore A, Birolo L, Giangrande C, Soccol VT, Pandey A, Soccol CR (2012). Characterization of laccase isoforms produced by Pleurotus ostreatus in solid state fermentation of sugarcane bagasse. Biores Technol 114: 735-739
[28] Ali HKQ, Zulkali MMD (2011). Design aspects of bioreactors for Solid-state Fermentation: A review. Chem Biochem Eng Q 25(2): 255-266
[29] Lane MD, Seelig B (2014). Advances in the directed evolution of proteins. Curr Opin Chem Biol 22: 129-136
[30] Montella S, Amore A, Faraco V (2015). Metagenomics for the development of new biocatalysts to advance lignocellulose saccharification for bioeconomic development. Crit Rev Biotechnol. DOI: 10.3109/07388551.2015.1083939
[31] Lambertz C, Garvey M, Klinger J, Heesel D, Klose H, Fischer R, Commandeur U (2014). Challenges and advances in the heterologous expression of cellulolytic enzymes: a review. Biotechnol Biofuels 7: 135-149
[32] Formenti LR, Nørregaard A, Bolic A, Hernandez DQ, Hagemann T, Heins AL, Larsson H, Mears L, Mauricio-Iglesias M, Krühne U, Gernaey KV (2014). Biotechnol J 9(6): 727-738
[33] Palmowski L, Muller J (1999). Influence of the size reduction of organic waste on their anaerobic digestion. In: Proceedings of the II international symposium on anaerobic digestion of solid waste. Barcelona; 15-17June 1999. p.137-144
[34] Mohan D, Pittman CU, Steele PH (2006). Pyrolysis of wood/biomass for bio-oil: a critical review. Energ Fuels 20: 848-889
[35] Karunanithy C, Muthukumarappan K (2011). Influence of extruder and feedstock variables on torque requirement during pretreatment of different types of biomass-a response surface analysis. Biosystems Engineering 109: 37-51
[36] López-Arenas T, Rathi P, Ramírez-Jiménez E, Sales-Cruz M (2010). Factors affecting the acid pretreatment of lignocellulosic biomass: Batch and continuous process. Comput Aided Chem Eng 28: 979-984
[37] Chang VS, Nagwani M, Kim CH, Holtzapple MT (2001). Oxidative lime pretreatment of high-lignin biomass poplar wood and newspaper. Appl Biochem Biotech 94: 1-28
[38] Mesa L, Gonzalez E, Cara C, González M, Castro E, Mussatto SI (2011). The effect of organosolv pretreatment variables on enzymatic hydrolysis of sugarcane bagasse. Chem Eng J 168: 1157-1162
[39] Mood SH, Golfeshan AH, Tabatabaei M, Jouzani GS, Najafi GH, Gholami M, Ardjmand M (2013). Lignocellulosic biomass to bioethanol, a comprehensive review with a focus on pretreatment. Renew Sust Energ Rev 27: 77-93
[40] Singh R, Shukla A, Tiwari S, Srivastava M (2014). A review on delignification of lignocellulosic biomass for enhancement of ethanol production potential. Renew Sust Energ Rev 32: 713-728
[41] Maurya DP, Singla A, Negi S (2015). An overview of key pretreatment processes for biological conversion of lignocellulosic biomass to bioethanol. 3 Biotech 5: 597-609
[42] Moreno AD, Ibarra D, Alvira P, Tomás-Pejó E, Ballesteros M (2015). A review of biological delignification and detoxification methods for lignocellulosic bioethanol production. Crit Rev Biotechnol 35(3): 342-54

[43] Reungsang A, Pattra S, Sittijunda S (2012). Optimization of key factors affecting methane production from acidic effluent coming from the sugarcane juice hydrogen fermentation process. Energies 5: 4746-4757
[44] Melikoglua M, Lina CSK, Webb C (2013). Stepwise optimisation of enzyme production in solid state fermentation of waste bread pieces. Food Bioprod Process 91: 638-646
[45] Gujarathia AM, Sadaphalb A, Bathec GA (2015). Multi-objective optimization of solid state fermentation process. Mater Manuf Process 30(4): 511-519
[46] Tian XF, Fang Z, Guo F (2012). Impact and prospective of fungal pre-treatment of lignocellulosic biomass for enzymatic hydrolysis. Biofuels Bioprod Bioref 6(3): 335-350
[47] Sindhu R, Binod P, Pandey A (2016). Biological pretreatment of lignocellulosic biomass - An overview. Bioresour Technol199: 76-82
[48] Huang C, Zong MH, Wu H, Liu QP (2009). Microbial oil production from rice straw hydrolysate by *Trichosporon fermentans*. Bioresour Technol 100(19): 4535-4538
[49] Brown ME, Chang MCY (2014). Exploring bacterial lignin degradation. Curr Opin Chem Biol 19: 1-7
[50] Heinfling A, Ruiz-Duefias FJ, Martinez MJ, Bergbauer M, Szewzyk U, Martinez AT (1998). A study on reducing substrates of manganese-oxidizing peroxidases from *Pleurotus eryngii* and *Bjerkundera adusta*. FEBS Len 428: 141-146
[51] Martínez ÁT, Ruiz-Dueñas FJ, Martínez MJ, del Río JC, Gutiérrez A (2009). Enzymatic delignification of plant cell wall: from nature to mill. Curr Opin Biotech 20: 348-357
[52] Ibarra D, Romero J, Martínez MJ, Martínez AT, Camarero S (2006). Exploring the enzymatic parameters for optimal delignification of eucalypt pulp by laccase mediator. Enzyme Microb Technol 39: 1319-27
[53] Kondo A, Ishii J, Harac KY, Hasunuma T, Matsuda F (2013). Development of microbial cell factories for bio-refinery through synthetic bioengineering. J Biotechnol 163: 204-216
[54] Kumar D, Murthy GS (2013). Stochastic molecular model of enzymatic hydrolysis of cellulose for ethanol production. Biotechnol Biofuel 6: 63-83
[55] Yang B, Dai Z, Ding SY, Wyman CE (2011). Enzymatic hydrolysis of cellulosic biomass. Biofuels 2(4): 421-450
[56] http://www.cazy.org/
[57] http://foly.esil.univ-mrs.fr/
[58] Van Dyk JS, Pletschke BI (2012). A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes-factors affecting enzymes, conversion and synergy. Biotechnol Adv 30: 1458-1480
[59] Hasunuma T, kazaki F, Okai N, Hara KY, Ishii J, Kondo A (2013). A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology. Biores Technol 135: 513-522
[60] Beg QK, Kapoor M, Mahajan L, Hoondal GS (2001). Microbial xylanases and their industrial applications: a review. Appl Microbiol Biotechnol 56(3-4): 326-38
[61] Hu J, Arantes V, Saddler JN (2011). The enhancement of enzymatic hydrolysis of lignocellulosic substrates by the addition of accessory enzymes such as xylanase: is it an additive or synergistic effect? Biotechnol Biofuels 5: 4:36-50
[62] Olofsson K, Bertilsson M, Lidén G (2008). A short review on SSF - an interesting process option for ethanol production from lignocellulosic feedstocks. Biotechnol Biofuels 1:7-20
[62] Tesfaw A, Assefa F (2014). Current trends in bioethanol production by *Saccharomyces cerevisiae*: substrate, inhibitor reduction, growth variables, coculture, and immobilization. International Scholarly Research Notices Volume 2014 (2014), Article ID 532852, 11 pages
[63] Chandel AK, Chandrasekhar G, Radhika K, Ravinder R, Ravindra P (2011). Bioconversion of pentose sugars into ethanol: A review and future directions. Biotechnol Mol Biol Rev 6(1): 8-20
[64] Scully SM, Orlygsson J (2015). Recent advances in second generation ethanol production by thermophilic bacteria. Energies 8: 1-30

[65] Dashtban M, Schraft H, Qin W (2009). Fungal bioconversion of lignocellulosic residues; opportunities & perspectives. Int J Biol Sci 5(6): 578-595
[66] Oberoi HS, Babbar N, Dhaliwal SS, Kaur U, Chadha BS, Bhargav VK (2012). Ethanol production from alkali-treated rice straw via simultaneous saccharification and fermentation using newly isolated thermotolerant *Pichia kudariavzavii*. Indian J Biotechnol 39: 557-66
[67] Amore A, Faraco V (2012). Potential of fungi as category I Consolidated BioProcessing organisms for cellulosic ethanol production. Renew Sust Energ Rev 16: 3286-3301
[68] Jouzani GS, Taherzadeh MJ (2015). Advances in consolidated bioprocessing systems for bioethanol and butanol production from biomass: a comprehensive review. Biofuel Res J 4(2): 152-195
[69] Liu S, Bischoff KM, Li Y, Cui F, Azaizeh H, Tafesh A. Production of value-added products by lactic acid bacteria. In Hou CT, Shaw J-F Editors. Biocatalysis and Biomolecular Engineering. John Wiley & Son; 2010. p. 421-435
[70] Axelsson L (2004). Lactic acid bacteria: classification and physiology. In S. Salminen, A. Von Wright, & A. Ouwehand (Eds.), Microbiological and functional aspects. New York: Marcel Dekker, Inc.
[71] Pedersen MB, Gaudu P, Lechardeur D, Petit MA, Gruss A (2012). Aerobic respiration metabolism in lactic acid bacteria and uses in biotechnology. Annu Rev Food Sci Technol 3(1): 37-58
[72] Bustos G, Moldes AB, Cruz JM, Domínguez JM (2005). Production of lactic acid from vine-trimming wastes and viticulture lees using a simultaneous saccharification fermentation. J Sci Food Agr 472: 466-472
[73] Marques S, Santos JAL, Gírio FM, Roseiro JC (2008). Lactic acid production from recycled paper sludge by simultaneous saccharification and fermentation. Biochem Eng J 41: 210-216
[74] Adhikari DK, Trivedi J, Agrawal D (2014). Consolidated bio processing of lignocellulosic biomass for l-lactic acid production. Patent number: WO 2014013509 A1
[75] Sandhu SK, Oberoi HS, Babbar N, Miglani K, Chadha BS, Nanda DK (2013). Two-stage statistical medium optimization for augmented cellulase production via solid-state fermentation by newly isolated *Aspergillus niger* HN-1 and application of crude cellulase consortium in hydrolysis of rice straw. J Agric Food Chem 61: 12653-12661
[76] Ortiz GE, Guitart ME, Cavalitto SF, Albertó EO, Fernández-Lahore M., Blasco M (2015). Characterization, optimization, and scale-up of cellulases production by *Trichoderma reesei* cbs 836.91 in solid-state fermentation using agro-industrial products. Bioprocess Biosyst Eng 38: 2117-2128
[77] Baldrian P, Valášková V (2008). Degradation of cellulose by basidiomycetous fungi. FEMS Microbiol Rev 32: 501-521
[78] Liu G, Qin Y, Li Z, Qu Y (2013). Development of highly efficient, low-cost lignocellulolytic enzyme systems in the post-genomic era. Biotechnol Adv 3: 962-975
[79] Rashid SS, Alam MZ (2013). Statistical optimization of the enzymatic saccharification of the oil palm empty fruit bunches. World Acad Sci Eng Technol 7: 253-258
[80] Jagtap SS, Dhiman SS, Jeya M, Kang YC, Choi J-H, Lee J-K (2012). Saccharification of poplar biomass by using lignocellulases from *Pholiota adiposa*. Biores Technol 120: 264-272
[81] Pandiyan K, Tiwari R, Singh S, Nain PKS, Rana S, Arora A, Singh SB, Nain L (2014). Optimization of enzymatic saccharification of alkali pretreated *Parthenium* sp. using response surface methodology. Enzyme Res, Article ID 764898, 8 pages
[82] Wang Z, Xu J, Cheng JJ (2011). Modeling biochemical conversion of lignocellulosic materials for sugar production: a review. Biores 6(4):5282-5306
[83] Khoo HH (2015). Review of bio-conversion pathways of lignocellulose to ethanol: sustainability assessment based on land footprint projections. Renew Sust Energ Rev 46: 100-119
[84] de Jong E, Jungmeier G. Biorefinery concepts in comparison to petrochemical refineries. In: Pandey A, Höfer R, Taherzadeh M, Nampoothiri I, Larroche C, editors. Industrial Biorefineries and White Biotechnology, vol. 1. Elsevier; 2015. p. 3-33

[85] United Nations Environment Programme (UNEP) and International Solid Waste Association (ISWA) (2015). Global Waste Management Outlook. Wilson DC (Ed) Authors: Wilson DC, Rodic L, Modak P, Soos R, Carpintero A, Velis CA, Iyer M and Simonett O. UNEP International Environment Technology Centre: Osaka, September 2015
[86] Velis CA (2015). Circular economy and global secondary material supply chains. Waste Manage Res 33(5): 389-391
[87] Kamm B, Kamm M (2004). Biorefinery - systems. Chem Biochem Eng 18(1):1-6
[88] Gavrilescu M (2014). Biorefinery systems: An overview. Bioenergy Research: Advances and Applications, chapter 14, p. 219-241
[89] Abraham TW, Höfer R. Lipid-based polymer building blocks and polymers. In: Matyjaszewski K, Möller M, editors. Polymer science: a comprehensive reference. McGrath JE, Hickner MA, Höfer R, editors. Polymers for a sustainable environment and green energy, vol. 10. Elsevier; 2012. p. 15-58
[90] Schaffenberger M, Ecker J, Koschuh W, Essl R, Mandl MG, Boechzelt HG, Steinmueller H, Schnitzer H (2012). Green biorefinery – production of amino acids from grass silage juice using an ion exchanger device at pilot scale. Chem Eng Trans 29: 505-510
[91] Antelo LT, de Hijas-Liste GM, Franco-Uría A, Alonso AA, Pérez-Martín RI (2014). Optimisation of processing routes for a marine biorefinery. Journal of Clean Prod 104: 489-501
[92] Jacquet N, Haubruge E, Richel A (2015). Production of biofuels and biomolecules in the framework of circular economy: A regional case study. Waste Manage Res 33(12): 1121-1126
[93] Renewables 2015 Global Status Report (Paris: REN21 Secretariat). ISBN 978-3-9815934-6-4
[94] Fava F, Totaro G, Diels L, Reis M, Duarte J, Carioca OB, Poggi-Varaldo HM, Ferreira BS (2015). Biowaste biorefinery in Europe: opportunities and research & development needs. New Biotechnol (32)1: 100-108
[95] Bayer EA, Chanzy H, Lamed R, Shoham Y (1998). Cellulose, cellulases and cellulosomes. Curr Opin Struct Biol 8: 548-557
[96] Ljungdahl LG (2008). The cellulase/hemicellulase system of the anaerobic fungus *Orpinomyces* PC-2 and aspects of its applied use. Ann N Y Acad Sci 1125: 308-321
[97] Sanchez C (2009). Lignocellulosic residues: biodegradation and bioconversion by fungi. Biotechnol Adv 27:185-194
[98] http://genome.jgi-psf.org/
[99] Giardina P, Autore F, Faraco V, Festa G, Palmieri G, Piscitelli A and Sannia G (2007). Structural characterization of heterodimeric laccases from *Pleurotus ostreatus*. App Microbiol Biotechnol 75:1293-1300
[100] Faraco V, Ercole C, Festa G, Giardina P, Piscitelli A and Sannia G (2008). Heterologous expression of heterodimeric laccases from *Pleurotus ostreatus* in *Kluyveromyces lactis*. App Microbiol Biotechnol 77:1329- 1335
[101] Lamb J, Loy T (2005). Seeing red: the use of Congo Red dye to identify cooked and damaged starch grains in archaeological residues. J Archaeol Sci 32(10): 1433-1440
[102] Florencio C, Couri S, Sanchez Farinas C (2012). Correlation between agar plate screening and solid-state fermentation for the prediction of cellulase productionb by Trichoderma strains. Enzyme Res, Volume 2012, Article ID 793708, 7 pages http://dx.doi.org/10.1155/2012/793708
[103] Ang TN, Ngoh GC, Chua AS (2011). A quantitative method for fungal ligninolytic enzyme screening studies. Asia-Pac. J Chem Eng 6: 589-595
[104] Kang SW, Park YS, Lee JS, Hong SI and Kim SW (2004). Production of cellulases and hemicellulases by *Aspergillus niger* KK2 from lignocellulosic biomass. Biores Technol 91: 153-156
[105] Ncube T, Howard RL, Abotsi EK, Jansen van Rensburg EL and Ncube I (2012). Jatropha curcas seed cake as substrate for production of xylanase and cellulase by *Aspergillus niger* FGSCA733 in solid-state fermentation. Ind Crop Prod 37: 118-123

[106] Ang SK, Shaza EM, Adibah YA, Suraini AA and Madihah MS (2013). Production of cellulases and xylanase by *Aspergillus fumigatus* SK1 using untreated oil palm trunk through solid state fermentation. Process Biochem 48(9):1293-1302
[107] Delabona P da Silva, Pirotaa RDPB, Codimaa CA, Tremacoldi CR, Rodrigues A and Farinas CS (2013). Effect of initial moisture content on two Amazon rainforest *Aspergillus* strains cultivated on agro-industrial residues: Biomass-degrading enzymes production and characterization. Ind Crop Prod 42: 236-242
[108] Dai X-J, LiU M-Q, Jin H-X and Jing M-Y (2011). Optimisation of solid-state fermentation of *Aspergillus niger* JL-15 for xylanase production and xylooligosaccharides preparation. Czech Journal of Food Science 29(5): 557-567
[109] Montibeller Weingartner V, de Souza Vandenberghe LP, Amore A, Soccol CR, Birolo L, Vinciguerra R, Salmon DNX, Rigon Spier M, Faraco V (2014). Characterization of hemicellulolytic enzymes produced by *Aspergillus niger* NRRL 328 under solid state fermentation on soybean husks. Biores 9(4): 7128-7140
[110] Rodríguez-Fernández DE, Rodríguez-León JA, de Carvalho JC, Sturm W and Soccol CR (2011). The behavior of kinetic parameters in production of pectinase and xylanase by solid-state fermentation. Biores Technol 102(22): 10657-10662
[111] Lee CK, Darah I and Ibrahim CO (2011). Production and optimization of cellulase enzyme using *Aspergillus niger* USM AI 1 and comparison with *Trichoderma reesei* via solid state fermentation system. Biotechnol Res Int 658493: 658496
[112] Mrudula S and Murugammal R (2011). Production of cellulase by *Aspergillus niger* under submerged and solid state fermentation using coir waste as a substrate. Braz J Microbiol 42: 1119-1127
[113] Bansal N, Tewari R, Soni R, Soni SK. Production of cellulases from *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen waste residues. Waste Manage 32: 1341-1346
[114] Kurt S, Buyukalaca S (2010). Yield performances and changes in enzyme activities of *Pleurotus* spp. (*P. ostreatus* and *P. sajor-caju*) cultivated on different agricultural wastes. Bioresour Technol 101(9): 3164-3169
[115] Kumaran S, Sastry CA and Vikineswary S (1997). Laccase, cellulase and xylanase activities during growth of *Pleurotus sajor-caju* on sago *hampas*. World J Microbiol Biot 13: 43-49
[116] Sathishkumar P, Murugesan K and Palvannan T (2010). Production of laccase from Pleurotus *florida* using agro-wastes and efficient decolorization of Reactive blue 198. J Basic Microb 50: 360-367
[117] El-Batal AI, ElKenawy NM, Yassin AS, Amin MA (2015). Laccase production by *Pleurotus ostreatus* and its application in synthesis of gold nanoparticles. Biotechnol Rep 5: 31-39
[118] Amore A, Pepe O, Ventorino V, Birolo L, Giangrande C, Faraco V (2013). Industrial waste based compost as a source of novel cellulolytic strains and enzymes. FEMS microbiology letters 339(2): 93-101
[119] Iandolo D, Piscitelli A, Sannia G and Faraco V (2011). Enzyme production by solid substrate fermentation of *Pleurotus ostreatus* and *Trametes versicolor* on tomato pomace. Appl Biochem Biotechnol 163: 40-5
[120] Ghose TK (1987). Measurement of cellulase activities. Pure & Appl Chem 59(2): 257-268
[121] Bailey MJ, Biely P, Poutanen K (1992). Interlaboratory testing of methods for assay of xylanase activity. J Biotech 23: 257-270
[122] Miller GL (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar
Anal Chem 31: 426-428
[123] Palmieri G, Giardina P, Bianco C, Scaloni A, Capasso A, Sannia G (1997). A novel white laccase from *Pleurotus ostreatus*. J Biol Chem 272. 31301-31307
[124] Laemmli UK (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685

[125] Amore A, Pepe O, Ventorino V, Birolo L, Giangrande C, Faraco V (2012). Cloning and recombinant expression of a cellulase from the cellulolytic strain *Streptomyces* sp. G12 isolated from compost. Microbial Cell Factories 11: 164-175
[126] Amore A, Parameswaran B, Kumar R, Birolo L, Vinciguerra R, Marcolongo L, Ionata E, La Cara F, Pandey A, Faraco V (2015). Application of a new xylanase activity from *Bacillus amyloliquefaciens* XR44A in Brewer's spent grain saccharification. J Chem Technol Biotechnol 90: 573-581
[127] Karp SG, Faraco V, Amore A, Birolo L, Giangrande C, Soccol VT, Pandey A, Soccol CR (2012). Characterization of laccase isoforms produced by *Pleurotus ostreatus* in solid state fermentation of sugarcane bagasse. Biores Technol 114: 735-739
[128] Mussatto SI, Dragone G, Roberto IC (2006). Brewers' spent grain: generation, characteristics and potential applications. J Cereal Sci 43(1): 1-14
[129] Kang Q, Appels L, Tan T, Dewil R (2014). Bioethanol from lignocellulosic biomass: current findings determine research priorities. The Scientific World Journal, Volume 2014, Article ID 298153, 13 pages

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Appendices

Appendix I

List of communications

1. Antonella Amore, **Rossana Liguori**, Parameswaran Binod and Vincenza Faraco. New technologies and tools for second generation bioethanol production: the BIOASSORT project. FISV2014, XIII Congress, book of abstracts and poster section. Pisa (Italy), 24th-27thSeptember 2014.

2. **R**ossana **Liguori**, Vincenza Faraco, Antonella Amore, Carlos Ricardo Soccol, VaneteThomaz Soccol, Adenise Lorenci Woiciechowski, Luciana Porto de Souza Vandenberghe and Binod Parameswaran. Screening for novel fungi producing ligninolytic, cellulolytic and xylanolytic activities. BioTech 2014 and 6th Czech-Swiss Symposium, book of abstracts and poster section. Prague (Czech Republic), 11th-14thJune 2014.

3. Vincenza Faraco, Antonella Amore, **R**ossana **Liguori**, Carlos Ricardo Soccol, VaneteThomaz Soccol, Adenise Lorenci Woiciechowski, Luciana Porto de Souza Vandenberghe and Binod Parameswaran. New microorganisms for wastes upgrading: the case of Organic Fraction of Municipal Solid Wastes and Brewer's spent grain within Bioassort project. BioTech 2014 and 6th Czech-Swiss Symposium, book of abstracts and oral presentation. Prague (Czech Republic), 11th-14thJune 2014.

Appendix II

List of publications

1. **R**ossana **Liguori**, Antonella Amore and Vincenza Faraco (2013). Waste valorization by biotechnological conversion into added value products. *Applied Microbiology and Biotechnology*, **97:** 6129-6147. DOI: 10.1007/s00253-013-5014-7

2. Antonella Amore*, SimonaGiacobbe*, **R**ossana **Liguori*** and VincenzaFaraco* (2014). (*These authors equally contributed to this manuscript). The second generation ethanol production. *Rendiconti Accademia Nazionale delle Scienze detta dei XL Memorie di Scienze Fisiche e Naturali*. ISBN 987-88-548-7171-7, XXXVII, 113-136.DOI: 10.4399/97888548717176

3. **R**ossana **Liguori**, Carlos Ricardo Soccol, Luciana Porto de Souza Vandenberghe, Adenise Lorenci Woiciechowski and Vincenza Faraco (2015). Second generation ethanol production from Brewers' Spent Grain. *Energies*, **8**: 2575-2586. DOI: 10.3390/en8042575

4. Salvatore Fusco, **R**ossana **Liguori**, Danila Limauro, Simonetta Bartolucci, She Qunxin and Patrizia Contursi (2015). Transcriptome analysis of *Sulfolobus solfataricus* infected with two related fuselloviruses reveals novel insights into the regulation of CRISPR-Cas system. *Biochimie*, **118:** 322-332. DOI: org/10.1016/j.biochi.2015.04.006

5. **R**ossana **Liguori**, Elena Ionata, Loredana Marcolongo, Luciana Porto de Souza Vandenberghe, Francesco La Cara and Vincenza Faraco (2015). Optimization of *Arundo donax* saccharification by(hemi)cellulolytic enzymes from *Pleurotus ostreatus*. *BioMed Research International*, 14 pages; Article ID 951871. DOI: dx.doi.org/10.1155/2015/951871

6. **R**ossana **Liguori**, Carlos Ricardo Soccol, Luciana Porto de Souza Vandenberghe, Adenise Lorenci Woiciechowski, Elena Ionata, Loredana Marcolongo and Vincenza Faraco (2015). Selection of the strain *Lactobacillus acidophilus* ATCC 43121 and its application to Brewers' Spent Grain conversion into lactic acid. *BioMed Research International*, 9 pages; Article ID 240231. DOI: http://dx.doi.org/10.1155/2015/240231

7. **R**ossana **Liguori**, Valeria Ventorino, Olimpia pepe and Vincenza Faraco (2016). Bioreactors for lignocellulose conversion into fermentable sugars for production of high added value products. *Applied Microbiology and Biotechnology*, *Appl Microbiol Biotechnol,* **100:** 597-611.DOI: 10.1007/s00253-015-7125-9

Appendix III

Experiences in foreign laboratories

- Stage at department of Engineering of Bioprocess and Biotechnology of **UFPR Institute** (University Federal do Paranà - **Curitiba**, **Brazil**) for selection of new ethanologenic, lactic, ligninolytic, cellulolytic and xylanolytic microorganisms for waste upgrading into high added value products such as lactic acid and ethanol (July-December 2013). Prof. Dr. Carlos Ricardo Soccol.

- Stage at department of Engineering of Bioprocess and Biotechnology of **TEC de Monterrey** (Centro de Biotecnologia-FEMSA of the InstitutoTecnologico y de Estudios Superiores de Monterrey - **Mexico**) for the development of new lignocellulosic residues pretreatment for waste upgrading into high added value products such as lactic acid and ethanol (August-October 2015). Prof. Dr.Sergio RománOthónSernaSaldivar.

Universidade Federal do Paraná
Setor de Tecnologia
Departamento de Engenharia de Bioprocessos e Biotecnologia
Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia

Curitiba, 14 de junho de 2013.

À
ROSSANA LIGUORI
UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II
DIPARTIMENTO DI SCIENZE CHIMICHE

Prezada Rossana,

Em virtude do desenvolvimento do projeto de pesquisa intitulado ***"Improvement of technologies and tools, e.g. biosystems and biocatalysts, for waste conversion to develop an assortment of high added value eco-friendly and cost-effective bio-products (BIOASSORT)"*** financiado pela *Commissione Europea Settimo Programma Quadro, Azioni Marie Curie* – ***schema International Research Staff Exchange - Contratto numero 318931***, coordenado pela *Università Deli Studi di Napoli Federico II*, sob a supervisão de Vicenza Faraco em colaboração com a Universidade Federal do Paraná, tenho o prazer em convidar-lhe a desenvolver experimentos relativos "**Engenharia Genética de Fungos Lignocelulolíticos**" previstos no projeto acima citado sob minha supervisão e da profª Vanete Thomaz Soccol no período de 1º de Julho a 1º de dezembro de 2013, na Divisão de Engenharia de Bioprocessos e Biotecnologia.

Durante o período de sua permanência no Brasil, os custos de passagem, estadia e alimentação serão custeados com os recursos financeiros aprovados no projeto BIOASSORT, os quais são administrados pela *Università Deli Studi di Napoli Federico II*.

Profº. Dr. Carlos Ricardo Soccol
Chefe da Divisão de Engenharia de Bioprocessos e Biotecnologia
Departamento de Engenharia de Bioprocessos e Biotecnologia
Universidade Federal do Paraná

Divisão de Engenharia de Bioprocessos e Biotecnologia
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Tecnológico de Monterrey

Montrerrey N.L., Mexico

October 12, 2015

To whom it may concern:

In the frame of the Bioassort Project entitled "*Improvement of Technologies and Tools e.g Biosystems and Biocatalysts for Waste Conversion to Develop an Assortment og High Added Eco-Friendly and Cost Effective Bio Products*" supported by grant 318931 of the Marie Curie International Research Staff Exchange Scheme Fellowship within the 7th European Framework Programme, coordinated by the University of Naples Federico II and leaded by Professor Vincenza Faraco,

Dott. ssa Rossana Liguori, PhD student in Biotecnologie Industriali e Molecolari XVIII ciclo, carried out part of her PhD activities at our Centro de Biotecnologia-FEMSA of the Instituto Tecnologico y de Estudios Superiores de Monterrey-Campus Monterrey from august 6 to October 12 of the present year.

Please do not hesitate to contact me if you require further information related to Rossana´s activities here in our institution.

Truly yours

Dr. Sergio O. Serna Saldivar

Professor and Research Leader

Center of Biotechnology - FEMSA

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CIDPRO
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Others publications

Appl Microbiol Biotechnol (2013) 97:6129–6147
DOI 10.1007/s00253-013-5014-7

MINI-REVIEW

Waste valorization by biotechnological conversion into added value products

Rossana Liguori · Antonella Amore · Vincenza Faraco

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Abstract Fossil fuel reserves depletion, global warming, unrelenting population growth, and costly and problematic waste recycling call for renewable resources of energy and consumer products. As an alternative to the 100 % oil economy, production processes based on biomass can be developed. Huge amounts of lignocellulosic wastes are yearly produced all around the world. They include agricultural residues, food farming wastes, "green-grocer's wastes," tree pruning residues, and organic and paper fraction of urban solid wastes. The common ways currently adopted for disposal of these wastes present environmental and economic disadvantages. As an alternative, processes for adding value to wastes producing high added products should be developed, that is the upgrading concept: adding value to wastes by production of a product with desired reproducible properties, having economic and ecological advantages. A wide range of high added value products, such as enzymes, biofuels, organic acids, biopolymers, bioelectricity, and molecules for food and pharmaceutical industries, can be obtained by upgrading solid wastes. The most recent advancements of their production by biotechnological processes are overviewed in this manuscript.

Keywords Enzymes · Biofuels · Organic acids · Biopolymers · Bioelectricity

Introduction

Fossil fuel reserves depletion, global warming, unrelenting population growth, and costly and problematic waste recycling call for renewable resources of energy and consumer products. As an alternative to the 100 % oil economy, production processes based on biomass can be developed by using the whole plant, thus providing the biorefinery concept. Each constituent of the residual biomass can be extracted and functionalized in order to produce non-food and food fractions, intermediate agro-industrial products, and synthons. Three major industrial domains can be concerned: molecules, materials, and energy. Using of residual biomass as raw material would simultaneously give the advantage of reducing externalities associated with waste disposal.

The biorefinery concept based on the use of the whole plant can evolve to a generalization. The reduction of the wastes or byproducts could be linked not only to one whole plant transformation but also to the use of several plants based on complementarities of different processes, different factories belonging sometimes to different companies up to the zero-waste biorefinery concept. The wastes and byproducts of the first one could be the starting material for the second one and a source of energy for the third one. Optimization of the flow of raw materials, of byproducts, and of energy between different industrial production units on a given site is a generalization of the concept of biorefinery through an actual "Industrial Metabolism" (Octave and Thomas 2009). The substitution of oil products by biobased products will develop a new bioeconomy and new industrial processes respecting the sustainable development concept. Industrial biorefinery will be linked to the creation of new processes based on the 12 principles of green chemistry (clean processes, atom economy, renewable feedstocks, etc.). Biotechnology, especially white biotechnology, will take a major part into these new processes with biotransformations (enzymology and microorganisms) and fermentation.

Huge amounts of lignocellulosic wastes are yearly produced all around the world. They include agricultural residues, food farming wastes, "green-grocer's wastes," tree pruning residues, and organic and paper fraction of urban solid wastes. The common ways currently adopted for disposal of these

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wastes include landfilling—having as the main disadvantage the high cost of transport; incineration—not convenient because of the low calorific value and high water content; and feeding for animals—having the limit that not every waste is appropriate for every animal. As an alternative, processes for adding value to wastes should be developed, that is the upgrading concept: adding value to wastes by production of a product with desired properties, and economic and ecological advantages (Laufenberg et al. 2003). A wide range of high added value products, such as enzymes, biofuels, organic acids, biopolymers, bioelectricity, and molecules for food and pharmaceutical industries, can be obtained upgrading solid wastes by biotechnological processes. The most recent advancements of their production are overviewed in this manuscript.

Biofuels from wastes

Bioethanol

The largest ethanol producers in the world are the USA, Brazil, and China. In 2009, the USA produced 39.5×10^9 l of ethanol using corn as a feedstock, while the second largest producer, Brazil, created about 30×10^9 l of ethanol using sugarcane (Bhatia et al. 2012). Bioethanol market is expected to reach 100×10^9 l in 2015 (Bhatia et al. 2012). In fact, in the USA, the Department of Energy has set a goal of 60 billion gallons of renewable fuels per year to be produced by 2030, while in the European Union there is a mandatory target to substitute 10 % of its transportation fuels with renewable fuels by 2020. However, ethanol production from this edible feedstock poses concerns about competition with food and feed supplies, and the only sustainable alternative substrate for ethanol production is lignocellulosic biomass.

Non-edible lignocellulosic residual biomasses attract keen interest as source for production and recovery of added value products such as fuel ethanol, due to their abundance, widespread distribution, and non-competitiveness with food supply.

Lignocellulose conversion into ethanol commonly involves i) a pretreatment to remove the barrier of lignin and expose plant cell wall polysaccharides, ii) enzymatic saccharification of sugars with a (hemi)cellulolytic enzyme cocktail, and iii) fermentation of the sugars with ethanologenic microorganisms. Numerous pretreatment strategies have been developed such as physical treatment, chemical treatment (alkaline or acid), biological treatment, physicochemical treatment, i.e., steam explosion, liquid hot water, ammonia fiber expansion, supercritical fluid treatment, and thermochemical treatment (Chundawat et al. 2011). After pretreatment, cellulose and hemicelluloses are hydrolyzed to monomeric sugars (hexoses and pentoses) using acid or enzymatic methods. Enzymatic hydrolysis by (hemi)cellulases is the preferred method because of the higher conversion yields and less corrosive and toxic conditions compared to the acid hydrolysis (Taherzadeh and Keikhosro 2007). Fermentation of all free sugars into ethanol is then carried out by yeasts or bacteria.

However, many challenges have still to be faced for improving the competitiveness of cellulosic ethanol production process. Particularly, the cost of enzymes used in the process is considered as one of the key bottlenecks for producing fuels and chemicals from lignocellulosic biomass. Several efforts are underway to reduce the cost of enzyme production. Some of the strategies include improving the performance of the enzymes by increasing the specific activity and thereby minimizing enzyme dosage by direct evolution and site-directed mutagenesis, reducing the cost of enzyme production by improving cellulase titers during fermentation by process engineering using cheap substrates including biomass, producing enzymes near biorefinery, or expressing enzymes in plants.

The enzymatic hydrolysis may take place in a separate step followed by fermentation called separate hydrolysis and fermentation (SHF) process, or it may take place together with the fermentation in a simultaneous saccharification and fermentation of hexoses (SSF) process or simultaneous saccharification and co-fermentation (SSCF) of both hexoses and pentoses (Fig. 1). The ultimate objective is a one-step consolidated bioprocessing (CBP) of lignocellulose to bioethanol (Amore and Faraco 2011; Olson et al. 2011), in which all the steps occur in a single reactor where a single microorganism or microbial consortium converts pretreated biomass into ethanol without added enzymes (Fig. 1).

Identification of the most appropriate lignocellulosic wastes to be used as feedstock has to be performed on a local basis taking climatic and logistic conditions, such as the availability and the current disposal methods, into account. For instance, as reported by Faraco and Hadar (2011), residues from cereal crops, olive trees, and tomato and grape

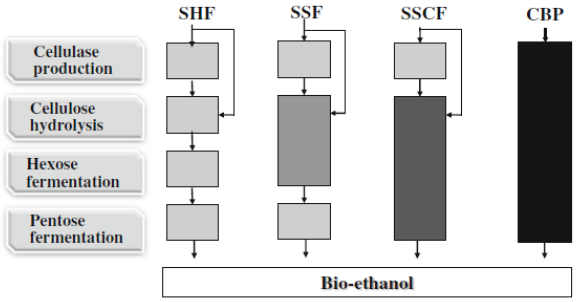

Fig. 1 Processes of bioethanol production. *SHF* separate hydrolysis and fermentation, *SSF* simultaneous hydrolysis and fermentation, *SSCF* simultaneous saccharification and co-fermentation of both hexoses and pentoses, *CBP* consolidated bioprocessing

processing are the most abundant lignocellulosic wastes in France, Italy, Spain, Turkey, and Egypt, where their use as raw materials for ethanol production could give rise to a potential production capacity of 13 Mtoe of ethanol. Due to the lack of sufficient amounts of agricultural residues in all of the other Mediterranean countries, use of the cellulosic content of municipal solid waste (MSW) as feedstock for ethanol fuel production is also proposed. A maximum potential production capacity of 30 Mtoe of ethanol could be achieved from 50 % of the 180 million tons of wastes currently produced annually in the Mediterranean Basin, the management of which has become a subject of serious concern.

Table 1 summarizes some examples of fermentation processes producing ethanol from wastes.

Several fruits processing wastes, such as banana peel (Joshi et al. 2001), mangoes (Reddy and Reddy 2007), and pineapple (Muttara and Nirmala 1982) have been successfully used as substrates for ethanol production. *Papaya* (pawpaw) agricultural waste, largely produced in Africa, has been used by Akin-osanaiye et al. (2008) as substrate for yeast fermentation, yielding a maximum of 5 % of ethanol after 24 h of simultaneous saccharification and fermentation by *Aspergillus niger* and *Saccharomyces cerevisiae*. Recently, grapes waste has been reported as a good substrate for ethanol production by *S. cerevisiae* strains as reported by Raikar (2012) (with an ethanol yield of 16 %) and Asli (2010) (with an ethanol yield of 95 g L^{-1}).

Wheat straw (Singh and Bishnoi 2012a; Passoth et al. 2013), rice straw (Singh and Bishnoi 2012b), oat, and barley straw (Hahn-Hägerdal et al. 2006) have been largely reported as a source for bioethanol production, and many efforts have been put on their pretreatment optimization for increasing accessibility of polysaccharides (Balan et al. 2009; Zhong et al. 2009). Corn stalks and soybean residues have also been reported as a source of fermentable sugars (Zaldivar et al. 2001; Yang et al. 2011).

Mutreja et al. (2011) investigated the effect of different pretreatment of eight lignocellulosic wastes as substrates for ethanol production, namely *Syzygium cumini* (jamun), *Azadirachta indica* (neem), *Saracens indica* (asoka), *Bambusa dendrocalmus* (bamboo), *Populus nigra* (poplar), *Achnatherum hymenoides* (wild grass), *Eucalyptus marginata* (eucalyptus), *Mangifera indica* (mango), and acid pretreatment of *S. cumini* (Jamun) at 30 °C giving a maximum ethanol yield of 1.42 g/L.

Singh and Jain (1995) reported cane molasses as a good substrate for ethanol production by batch fermentation.

Use of MSW is a promising strategy to meet the world energy needs and reduce GHG emissions, as well. Particularly, the replacement of biomass with the biodegradable portion of MWS (BMSW) can bring environmental advantages in waste management, carbon dioxide cut, water quality and quantity control, land use, and biodiversity (Li 2008). In a previous study, Li et al. (2007) showed the production of about 52 % of fermentable glucose from BMSW. Recently, biodegradable wastes, such as kitchen waste, garden waste, and paper waste, have been shown suitable substrates for ethanol production, yielding about 90 % of glucose under optimized conditions (Li 2008). As for the use of lignocellulosic wastes, the use of BMSW as a

Table 1 Examples of fermentation processes producing ethanol from wastes

Organism	Waste	Reference
Saccharomyces cerevisiae and *Aspergillus niger*	Papaya (pawpaw) agricultural waste	Akin-osanaiye et al. (2008)
Saccharomyces cerevisiae SC1	Grapes waste	Raikar (2012); Asli (2010)
Wickerhamomyces anomalus and *Scheffersomyces stipitis*	Wheat straw	Passoth et al. (2013)
Aspergillus flavus and *Trichoderma reesei*	Wheat straw	Singh and Bishnoi (2012a)
Saccharomyces cerevisiae 424A(LNH-ST)	Rice straw	Zhong et al. (2009)
Flocculating yeast	Waste banana peels	Joshi et al. (2001)
Zymomonas mobilis	Cane molasses	Singh and Jain (1995)
Saccharomyces cerevisiae	*Syzygium cumini* (jamun), *Azadirachta indica* (neem), *Saracens indica* (asoka), *Bambusa dendrocalmus* (bamboo), *Populus nigra* (poplar), *Achnatherum hymenoides* (wild grass), *Eucalyptus marginata* (eucalyptus), *Mangifera indica* (mango)	Mutreja et al. (2011)
Saccharomyces cerevisiae 101 (wine strain)	Six varieties of mangoes	Reddy and Reddy (2007)
Clostridium phytofermentans	Switchgrass	Jain et al. (2013)
Phlebia sp. MG-60	Woody biomass	Kamei et al. (2012)

resource for bioethanol production is an advantageous route, leading to reduction of waste going to landfill or incineration, and to whole reduction of greenhouse gas emissions.

As examples of waste conversion into ethanol by CBP, the effectiveness of *Clostridium phytofermentans* solid-state fermentation in directly converting switchgrass into ethanol was evaluated by Jain et al. (2013). *C. phytofermentans* was chosen for the consolidated bioprocessing of biomass since it is able to grow on diverse substrates through the production of 161 enzymes involved in the breakdown or modification of complex carbohydrates (Cantarel et al. 2009), and it produces ethanol as its major product along with a lesser quantity of acetate (Warnick et al. 2002; Weber et al. 2010). The study shows that, during the solid-state fermentation, the maximum concentration of ethanol, 58.14 mM was measured on the 12th day of incubation. The acetate and ethanol concentrations were similar during the initial 6 days of fermentation, but between days 6 and 14, the ethanol concentration increased significantly and surpassed the highest acetate concentration of 27.56 mM. Differently, during the submerged-state fermentation, the production of acetate (5 mM) and ethanol (27.56 mM) concentrations increased until the sixth day but did not increase further.

Kamei et al. (2012) reported ethanol production from woody biomass using a single microorganism without addition of chemicals or enzymes. They propose a new process of unified aerobic delignification and anaerobic saccharification and fermentation of wood by the white-rot fungus *Phlebia* sp. MG-60. This fungus is able to selectively degrade lignin under aerobic solid-state fermentation conditions, and to produce ethanol directly from delignified oak wood under semi-aerobic liquid culture conditions. After 56 days aerobic incubation, 40.7 % of initial lignin and glucan were degraded. Then under semi-aerobic conditions without the addition of cellulase, 43.9 % of theoretical maximum ethanol was produced after 20 days.

Biobutanol

Butanol is a product of ABE (acetone butanol ethanol) fermentation (Gottschalk 1986), and it represents an excellent chemical feedstock (in the plastics industry), and more importantly, a superior fuel compared to ethanol (Pfromm et al. 2010), since it is less corrosive, less hygroscopic, and tolerates water contamination better; it is less evaporative and explosive due to its lower vapor pressure; it has 30 % higher energy density than ethanol; it can be mixed in higher ratios with gasoline in existing cars without retrofitting the engine (Fatehi 2013). Butanol can be produced by a variety of microorganisms and the most commonly used strains are *Clostridium acetobutylicum* and *Clostridium beijerinckii* (Durre 1998; Qureshi et al. 2006).

Table 2 summarizes some examples of fermentation processes producing biobutanol from wastes.

In an attempt to reduce the cost of butanol production by fermentation, the use of a variety of low-cost feedstocks has been investigated such as molasses (Fan et al. 2010), sago starch (Madihah et al. 2001), wheat bran (Liu et al. 2010), barley straw hydrolysate (Qureshi et al. 2010), palm oil waste (Lee et al. 1995), and lignocellulosic materials such as wheat straw, cane molasses, corn, and switchgrass (Cho et al. 2009). Raganati et al. (2013) studied the butanol production by *C. acetobutylicum* DSM 792 fermentation on unsupplemented cheese whey. The conversion was successfully carried out in a biofilm packed bed reactor for more than 3 months. Under optimized conditions, the performances were a butanol productivity of 2.66 g/L/h, a butanol concentration of 4.93 g/L, a butanol yield of 0.26 g/L, and a butanol selectivity of the overall solvents production 82 wt.%. Ni et al. (2012) used cane molasses as substrate in ABE production by *Clostridium saccharobutylicum* DSM 13864. Under optimum conditions, total solvent of 19.80 g/L (13.40 g/L butanol) was reached after 72 h of fermentation in an Erlenmeyer flask. In a 5-L bioreactor, total solvent of 17.88 g/L was attained after 36 h of fermentation, and the productivity and yield were 0.50 g/L/h and 0.33 g ABE/g sugar consumption, respectively. To further enhance the productivity, a two-stage semi-continuous fermentation process was steadily operated for 8 days (205 h, 26 cycles) with average productivity (stage II) of 1.05 g/L/h and cell concentration (stage I) of 7.43 OD660, respectively. The average batch fermentation time (stage I and II) was reduced to 21–25 h with average solvent of 15.27 g/L.

Claassen et al. (2000) described the results obtained with fermentation of hydrolysate from domestic organic waste produced using Masonite steam explosion and enzymatic hydrolysis. *C. acetobutylicum* DSM 1731 *C. beijerinckii* B-592 and *Clostridium* LMD 84.48 produced 1.5 and 0.9 g/L ABE and 1.9 g/L ABE, respectively, from this hydrolysate without further supplementation. Incubation with two-fold concentrated hydrolysate completely impaired ABE production. After removal of unspecific inhibiting components, the yield of ABE production by *C. acetobutylicum* DSM 1731 increased about three-fold as compared to the non-treated hydrolysate. Similarly, Qureshi et al. (2010) studied the increase in butanol production after removal of inhibitors generated from the pretreatment of substrate. The fermentation of dilute sulfuric acid barley straw hydrolysate (BSH; undiluted/untreated) by *C. beijerinckii* P260 resulted in the production of 7.09 g/L ABE, an ABE yield of 0.33, and productivity of 0.10 g/L/h. This level of ABE is much less than that observed in a control experiment where glucose was used as a substrate (ABE yield of 0.41 and productivity of 0.31 g/L/h) suggesting the toxicity of BSH which was then reduced by treating it with lime [$Ca(OH)_2$]. The treated BSH resulted in the production of 26.64 g/L ABE. The presence of phenolic inhibitors has been detected also in pretreated fiber-rich agricultural biomass, causing a reduction

Table 2 Examples of fermentation processes producing biobutanol from wastes

Organism	Waste	Reference
Clostridium beijerinckii BA101	Medium based on corn steep water	Parekh and Blaschek (1999)
Clostridium saccharobutylicum DSM 13864	Cane molasses	Ni et al. (2012)
Clostridium acetobutylicum P260	Corn fiber xylan	Qureshi et al. (2006)
Clostridium acetobutylicum 2N	Sugar beet molasses	Fan et al. (2010)
Clostridium acetobutylicum	Gelatinized sago starch-derived sugars	Madihah et al. (2001)
Clostridium beijerinckii ATCC 55025	Wheat bran	Liu et al. (2010)
Clostridium beijerinckii P260	Dilute sulfuric acid barley straw hydrolysate	Qureshi et al. (2010)
Clostridium saccharoperbutylacetonicum N1-4	Palm oil waste	Lee et al. (1995)
Clostridium beijerinckii National Collection of Industrial and Marine Bacteria Ltd. 8052	Lignocellulosic hydrolysates	Cho et al. (2009)
Clostridium acetobutylicum DSM 792	Unsupplemented cheese whey	Raganati et al. (2013)
Clostridium acetobutylicum DSM 1731, *Clostridium beijerinckii* B-592, and *Clostridium* LMD	Hydrolysate from domestic organic waste	Claassen et al. (2000)
Clostridium beijerinckii BA101	Fiber-rich agricultural biomass	Ezeji et al. (2007)
Clostridium saccharoperbutylacetonicum N1-4	Palm oil mill effluent	Hipolito et al. (2008)

of 30 % yield of ABE by *C. beijerinckii* BA101 (Ezeji et al. 2007).

Biohydrogen

Hydrogen is becoming one of the most favorable new energy carriers because it is clean, recyclable, and can be used in fuel cells to directly generate electricity (Das and Veziroğlu 2001).

Fermentative production of hydrogen results from the fermentative conversion of organic substrate to biohydrogen manifested by a diverse group of bacteria using multi-enzyme systems involving three steps similar to anaerobic conversion. Dark fermentation reactions do not require light energy, so they are capable of constantly producing hydrogen from organic compounds throughout the day and night (Hussy et al. 2003). Photofermentation differs from dark fermentation because it only proceeds in the presence of light (Basak and Das 2006). Fermentative hydrogen production can be done using direct biophotolysis by green algae, indirect biophotolysis by cyanobacteria, photofermentation by anaerobic photosynthetic bacteria, and dark fermentation by anaerobic fermentative bacteria.

In recent works, it is indicated that photosynthetic bacteria can utilize several different waste materials as carbon sources for hydrogen production allowing, in the same time, the reduction of wastes. Table 3 summarizes some examples of fermentation processes producing biohydrogen from wastes.

Besides wastewaters of sugar refinery (Yetis et al. 2000), milk industry (Türkarslan et al. 1998), tofu industry (Zhu et al. 1999), and lactic acid fermentation plant (Sasikala and Ramana 1991), the main solid wastes usable as feedstock for hydrogen production include sewage sludge (Sunita and Mitra 1993) and municipal solid wastes (Fascetti et al. 1998). Biohydrogen produced from the hydrolyzate of cellulosic feedstock represents a promising candidate as a high efficiency energy carrier to supply future needs. Monlau et al. (2012) have determined which compositional (lignin, cellulose, hemicelluloses, total uronic acids, proteins, and soluble sugars) and structural (crystallinity) features of 20 kinds of lignocellulosic substrates can have an effect on biohydrogen production using predictive partial least square (PLS) models. They found that soluble sugars, namely non-structural carbohydrates present in the untreated substrates, had a significant positive effect on biohydrogen production because the hydrogen yield enhancement is correlated to an increase of soluble sugar content of the substrate. A pre-treatment of lignocellulosic substrates would enhance the biohydrogen production, leading to the solubilization of holocelluloses and increasing the soluble sugars fraction.

Fermentation technology for lignocellulosic hydrogen production can consist of a direct process in which cellulose hydrolysis and hydrogen production take place in the same reactor, or a two-stage process, in which cellulose is hydrolyzed in the first stage followed by dark hydrogen fermentation for hydrogen production in the second stage (Fig. 2).

The direct process is actually used for the conversion of agricultural wastes and wood into biohydrogen, and it is cheaper and more commercially feasible, but it needs choosing

Table 3 Examples of fermentation processes producing biohydrogen from wastes

Organism	Waste	Reference
Clostridium thermocellum ATCC 27405	Hulls	Magnusson et al. (2008)
Caldicellulosiruptor saccharolyticus (dark fermentation step) and *Rhodobacter capsulatus* (DSM1710) (photofermentation step)	Potato steam peels hydrolysate and molasses	Özgür et al. (2010)
Rhodobacter sphaeroides O.U.001	Waste water from milk industry	Türkarslan et al. (1998)
Rhodobacter sphaeroides O.U.001	Tofu wastewater	Zhu et al. (1999)
Rhodobacter sphaeroides O.U.001	Waste water of a lactic acid fermentation	Sasikala and Ramana (1991)
Rhodopseudomonas	Sewage and wastewater	Sunita and Mitra (1993)
Rhodobacter sphaeroides RV	Selected municipal solid wastes (lactate containing solutions derived from the acidogenesis)	Fascetti et al. (1998)

a microorganism able to produce cellulolytic enzymes and hydrogen simultaneously and it is less efficient due to the need to use the same temperature condition for cellulose degradation and dark H_2 fermentation. Many reports indicate that thermophilic microorganisms, such as *Thermococcus kodakaraensis*, *Clostridium thermolacticum*, and *Clostridium thermocellum* (Kanai et al. 2005; Liu et al. 2008) are suitable for biohydrogen production since they have the ability to produce cellulolytic enzymes and hydrogen simultaneously. It has been reported that *C. thermocellum* ATCC 27405 could use the hulls as the carbon source to produce hydrogen without any pretreatment, reaching the maximum hydrogen yield of 0.22 mol H_2/mol glucose (Magnusson et al. 2008).

In a two-stage process, after solubilizing cellulosic biomasses by hydrolysis, hydrogen fermentation would be easier to perform in a continuous process (Datar et al. 2007; Lo et al. 2010a). Since the theoretical hydrogen yield of the dark fermentation is usually within the range of 3–4 mol H_2/mol hexose and the system also produces 50–60 % of CO_2 in the biogas, actually, the best biohydrogen production system, which has high yield and low CO_2 emission, is based on the combination of the dark fermentation followed by photofermentation. The organic acids produced during the dark fermentation can be used by purple non-sulfur bacteria to produce hydrogen; this allows obtaining eight further moles of hydrogen per mole of hexose. Thus, a combination of dark and light fermentation is theoretically capable of producing the maximum yield of 12 mol of hydrogen per mole of hexose (Guwy et al. 2011). There are a lot of factors that significantly affect the efficiency of the combined process such as the presence of fixed nitrogen and the concentration and type of organic acids available (Akköse et al. 2009; Kim et al. 2006; Chen et al. 2010; Uyar et al. 2009; Su et al. 2009).

Photofermentative hydrogen production on effluents of thermophilic dark fermentations on potato steam peels (PSP) hydrolysate and molasses was investigated by using the

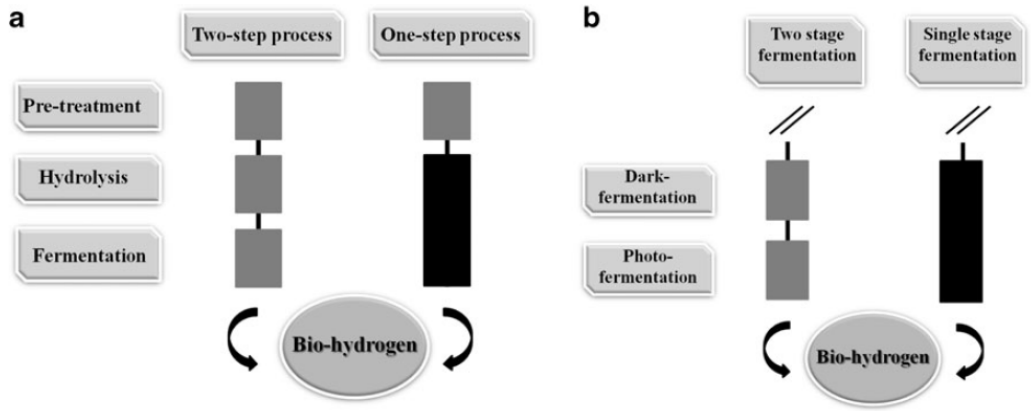

Fig. 2 Processes of biohydrogen production. **a** One-step process in which cellulose hydrolysis and hydrogen production take place in the same reactor; two-stage process, in which cellulose is hydrolyzed in the first stage followed by dark hydrogen fermentation for hydrogen production in the second stage. **b** Bioreactor design for fermentation: single stage in which light and dark fermentation are conducted in the same vessel or two stage in which dark fermentation and photofermentation are conducted in separate vessels

extreme thermophile *Caldicellulosiruptor saccharolyticus* in the dark fermentation step, and *Rhodobacter capsulatus* (DSM1710) in the following photofermentation step. To increase the hydrogen yields, it was necessary to adjust the initial acetate concentration in the dark fermentor effluents by dilution before using them in the photofermentation; the hydrogen yields (moles H_2 per mole consumed hexose) obtained on potato steam peels hydrolysate and molasses are 3.91 and 5.81, respectively (Özgür et al. 2010).

When considering the integration of dark fermentative hydrogen production and photofermentation, the bioreactor design is of considerable importance. Bioreactor designs are essentially of two types: single stage or two stages. In the latter system, dark fermentation and photofermentation are conducted in separate vessels; this allows optimizing the fermentation environment for each stage of biohydrogen production, but at the same time, increases the space requirements and operating costs rather than the single-stage system in which light and dark fermentation are conducted in the same vessel. On the other hand, the disadvantage of the single-stage system is the impossibility to optimize the environmental conditions for each phase (Argun and Kargi 2010). Actually, many researches are needed to optimize the bioH_2 production from waste in terms of production cost and efficiency. For example, in a recent study, a novel process integrating sequential dark photo fermentation (SDPF) and microalgae photoautotrophic process was proposed (Lo et al. 2010b). This process was shown to produce biohydrogen with a high-yield (up to 6.5 mol H_2/mol hexose) and low CO_2 emission (since all the CO_2 produced from fermentation was completely consumed by microalgae growth).

Biogas

Biogas is produced by the anaerobic digestion or fermentation of biodegradable materials, and it comprises primarily methane (CH_4) and carbon dioxide (CO_2) and may have small amounts of hydrogen sulfide (H_2S), moisture, and siloxanes (Schink 1997).

Wastes are suitable substrates for production of biogas in anaerobic digestion treatments. Table 4 summarizes some examples of fermentation processes producing biogas from wastes.

Initially, the emphasis was focused on anaerobic digestion of municipal solid waste (Braber 1995; Kiely et al. 1997), but also lignocellulosic waste can be used to obtain biogas-rich methane, although this waste conversion process requires, in addition, an initial pretreatment. The enzymatic pretreatment of sugar beet pulp, that has high fiber content, improves biogas yield from anaerobic fermentation (Ziemiński et al. 2012). Besides producing biogas, the anaerobic biological treatment decreases and stabilizes solid wastes volume generating a nutrient-rich digestate that can be used as fertilizer (Stroot et al. 2001). For these reasons, several studies, not only on laboratory scale but also on pilot- and full-scale anaerobic digestion for the treatment of organic solid waste, have already been reported (Igoni et al. 2008; Rao and Singh 2004; Lopez and Espinosa 2008; Elango et al. 2007; Fernandez et al. 2008). In general, the process is divided in three phases: hydrolysis, acidogenesis/acetogenesis, and methane production (Schink 1997). It was demonstrated that the composition and diversity of the microbial community have a great impact on the biogas yield. In fact, the process efficiency can be influenced by the variations in the microbial community that occur during the ongoing fermentation. Moreover, molecular analyses have shown the presence of numerous unknown bacteria, which may have an important influence on the degradation process (Weiland 2010). Furthermore, the efficiency of anaerobic digestion is improved combining two or more different types of feedstock (Álvarez et al. 2010) that allow balancing nutrients (C/N ratio and macro- and micronutrients) and diluting inhibitors/toxic compounds (Hartmann et al. 2004; Sosnowski et al. 2003). As a matter of fact, increased methane yields and volumetric productivities were observed in co-digestion of food waste with yard waste at specific ratios by Brown and Li (2013). Moreover, their study showed increased methane yields and volumetric productivities as the percentage of food waste was increased to 10 % and 20 % of the substrate at feedstock/effluent ratios (an operating parameter that measures the amount of substrate to the amount of inoculum) of 2 and 1, respectively. Zhang at al. (2013) studied the anaerobic co-digestion of food waste and cattle manure, and both batch and semi-continuous tests indicated that the total methane production is enhanced in co-digestion, with an optimum food waste to cattle manure ratio of 2. At this ratio, the total methane production in co-digestion increased by 55.2 % in semi-continuous mode and by 41.1 % in batch tests.

Organic acids from wastes

Bioconversion of lignocellulosic biomass could make a significant contribution to the production of organic chemicals. A wide range of organic acids can be produced by bacterial metabolism (Gottschalk 1986). Table 5 summarizes some examples of fermentation processes producing organic acids from wastes.

Volatile fatty acids such as acetic acid, propionic acids, oxalic acid, and butyric acid have been obtained from cereal hulls (Jin et al. 2002, 2004, 2006), bagasse residues (Henrique et al. 2005), food wastes (Lim et al. 2008), and sisal leaf decortications residues (Mshandete et al. 2008). In addition, lactic acid has been produced from waste sisal stems (Muruke

Table 4 Examples of fermentation processes producing (biogas) methane (CH_4) and carbon dioxide (CO_2) from wastes

Biosystem	Waste	Reference
Granular biomass from a pilot hybrid reactor treating wine waste and from an IC reactor treating brewery wastewater was used as inoculum	Pig manure and fish waste	Álvarez et al. (2010)
Effluent from a mesophilic liquid AD system fed with food wastes, fats, oils, and greases, and sewage sludge was used as inoculum	Yard waste	Brown and Li (2013)
Anaerobically treated activated sludge was used as inoculum	Cattle manure and food waste	Zhang et al. (2013)
Pig slurry	Organic fraction of municipal solid waste	Kiely et al. (1997)
Anaerobic sludge derived from an agricultural biogas	Sugar beet pulp and spent hops	Ziemiński et al. (2012)
Anaerobic sludge from a sewage sludge digester and/or cattle manure	Organic fraction of municipal solid waste, primary sludge, and waste activated sludge	Stroot et al. (2001)
Thickened excess activated sludge	Sewage sludge and organic fraction of municipal solid wastes	Sosnowski et al. (2003)
Seed inoculum collected from a nearby Khadi and Village Industries Commission cattle dung based biogas plant	Food wastes emanating from fruit and vegetable markets, households, hotels, and juice centers	Rao and Singh (2004)
Sewage contains organic solids and methanogenic bacteria	Municipal solid waste and domestic sewage	Elango et al. (2007)
Mesophilic sludge	Organic fraction of municipal solid waste	Fernandez et al. (2008)

et al. 2006), sugarcane bagasse (Adsul et al. 2007), and kitchen waste (Ohkouchi and Inoue 2007) by using *Lactobacillus* isolates.

Furthermore, formic acid, levulinic acid, citric acid, valeric acid, caproic acid, and vanillic acid are obtainable from bioprocessing of lignocellulosic waste (Olson 2001; Chaudhary and Sharma 2005; Mshandete et al. 2008; Ibrahim et al. 2008).

Abdel-Rahman et al. (2011) reviewed the advantages and limits of lactic acid production from several lignocellulose materials by simultaneous saccharification and fermentation. Wood hydrolysate (Wee et al. 2004), wheat bran hydrolysate (Givry et al. 2008), cassava bagasse (John et al. 2006), soft wood (Iyer et al. 2000), and sugarcane bagasse (Adsul et al. 2007) are some examples of lignocellulosic wastes investigated for lactic acid production by different *Lactobacillus* strain, yielding lactic acid in the range of 0.5–0.9 g per gram of substrate consumed. Due to the recalcitrance of lactic acid bacteria to process lignocelluloses hydrolyzates, new strains are being used for lactic acid production from this feedstock. Recently, a *Pediococcus pentosaceus* has been shown able to produce lactic acid from lignocellulose-derived mixed sugars including glucose, mannose, galactose, arabinose, and xylose (Adler et al. 2011). Guo et al. (2010) studied the production of lactic acid using corncob hydrolysates for the growth of *Rhizopus oryzae*, yielding 355 g lactic acid per kilogram corncobs, after 72 h incubation.

Table 5 Examples of fermentation processes producing organic acids

Biosystem	Waste	Product	Reference
Activated sludge inoculum (from an activated sludge process of a municipal wastewater treatment plant)	Sisal leaf decortications residues	Volatile fatty acids (organic acids)	Mshandete et al. (2008)
Lactobacillus sp.	Waste sisal stems	Lactic acid (organic acids)	Muruke et al. (2006)
Lactobacillus delbrueckii mutant Uc-3	Waste sugarcane bagasse	Lactic acid (organic acids)	Adsul et al. (2007)
Lactobacillus manihotivorans LMG18011	Fresh kitchen wastes from the cafeteria	Lactic acid (organic acids)	Ohkouchi and Inoue (2007)
Enterococcus faecalis	Wood hydrolysate	Lactic acid (organic acids)	Wee et al. (2004)
Lactobacillus bifermentans	Wheat bran hydrolysate	Lactic acid (organic acids)	Givry et al. (2008)
Lactobacillus sp.	Cassava bagasse	Lactic acid (organic acids)	John et al. (2006a)
Lactobacillus sp.	Soft wood	Lactic acid (organic acids)	Iyer et al. (2000)
Pediococcus pentosaceus	Lignocellulose-derived mixed sugars	Lactic acid (organic acids)	Adler et al. (2011)
Rhizopus oryzae	Corncob hydrolysates	Lactic acid (organic acids)	Guo et al. (2010)

Biopolymers from wastes

The potential of biodegradable polymers and particularly those obtained from agro-resources has long been recognized. Biodegradable plastics made from renewable resources can reduce petroleum consumption and decrease plastic waste disposal issues, since they are biodegradable in soil, compost, and marine environments. The so-called "agro-polymers" can be used in food industry and packaging, replacing conventional plastic materials. Polyhydroxybutyrate and polyhydroxyalkanoate are the major biodegradable polymers produced via biotechnological processes (Ojumu et al. 2004; Leaf and Srienc 1998). Polylactic acid is a biodegradable polymer composed of lactic acid monomers arising from lignocellulose as previously reported.

Table 6 summarizes some examples of fermentation processes producing biopolymers from wastes.

As reviewed by Mtui et al. (2009), many lignocellulosic materials such as domestic and agro-industrial residues have been investigated for production of biopolymers with improved properties such as biodegradability, mechanical strength, thermal stability, electrical conductivity, and recyclability.

Polyhydroxybutyrate is attracting the industry attention due to the similar biodegradability characteristics to those of conventional thermoplastics. With the tools of life cycle assessment (LCA), much effort is devoted to quantifying the environmental impact and feasibility of processes for production of this biobased-polymeric material (Sudesh and Iwata 2008). For instance, a recent LCA study explored fossil energy profiles of current and future scenarios for the production of the co-biopolymer polyhydroxybutyrate-valerate (PHBV), showing that the use of corn farming and corn-glucose for this polymer production strongly supports the moving to biopolymer production systems independent of fossil fuels (Guo et al. 2012).

A broad range of waste and surplus materials have been exploited for the bio-mediated production of polyhydroxyalkanoate biopolymer, as described by Koller et al. (2010). Starch-based residues, molasses, lignocellulose hydrolyzates, whey, glycerol liquid phase (from biodiesel production), and waste lipids can easily enter the metabolic pathway of PHA production.

The first study on PHA biosynthesis from the pentoses xylose and arabinose stemming from the hemicellulose fraction of poplar wood was done by Bertrand et al. (1990) with *Pseudomonas pseudoflava* ATCC 33668. Keenan et al. (2006) reported PHA biosynthesis in shaking flask cultures of *Burkholderia cepacia* grown on 2.2 % (*w*/*v*) xylose derived from forest biomass produced different P(3HB-*co*-3HV) copolyesters ranging from 1.3 to 4.2 g l^{-1}.

The production of biobased polymers from cheap alternative raw materials is strongly growing in recent years, as shown by the increasing number of manuscripts regarding the use of molasses, whey, lignocelluloses, fats and oils, glycerol, and wastewater for the development of biorefinery which combines biofuel production and high added value bio-products (Cha et al. 2007; Dumbrepatil et al. 2008; Kucukasik et al. 2009; Moralejo-Gárate et al. 2011; Koller et al. 2012; Naranjo et al. 2013).

Bioelectricity from wastes

Microbial fuel cells (MFCs) are a new idea in biological wastes treatment for conversion of biomass into electricity using their metabolic pathway (Rabaey and Verstraete 2005). MFC is a hybrid bioelectrochemical device which directly converts energy deposited in chemical bonds of substrate to electrical energy through bioelectrochemical reactions mediated by microorganisms. They extract energy necessary to build biomass (anabolic process) from redox reactions (catabolism) through electron donor/acceptor conditions. The potential (biologically mediated) developed between the bacterial metabolic activity [series of redox reactions generate electrons (e^-) and protons (H^+)] separated by a membrane manifests generation of bioelectricity (Venkata Mohan and Chandrasekhar 2011). MFCs have some advantages over the technologies currently used for generating energy from organic matter. First, the direct conversion of substrate energy to electricity enables high

Table 6 Examples of fermentation processes producing biopolymers

Biosystem	Waste	Product	Reference
Pseudomonas pseudoflava ATCC 33668	Hemicellulose fraction of poplar wood	Polyhydroxyalkanoate	Bertrand et al. (1990)
Burkholderia cepacia	Xylose derived from forest biomass	Polyhydroxyalkanoate	Keenan et al. (2006)
Cordyceps sinensis 16	Molasses	Exo-biopolymer	Cha et al. (2007)
Microbial community engineering	Glycerol	Polyhydroxyalkanoate	Moralejo-Gárate et al. (2011)
Bacillus megaterium	Glycerol	Polyhydroxybutyrate	Naranjo et al. (2013)
Saccharophagus degradans	Cellulosic waste from tequila bagasse	Polyhydroxyalkanoate	Alva Munoz and Riley (2008)
Alcaligenes latus DSM 1124	Soya wastes from a soya milk dairy and malt wastes from a beer brewery plant	Polyhydroxyalkanoate	

conversion efficiency, about eight times as high as conventional hydrogen production technologies (Yue and Lowther 1986). Second, MFCs operate efficiently at room temperature. Third, an MFC does not require gas treatment because the off-gases of MFCs are enriched in carbon dioxide, and fourth, MFCs do not need energy input for aeration provided the cathode is passively aerated (Lui et al. 2004). The efficiency and economic potency of converting organic wastes to bioenergy depend on the characteristics and chemical components of the waste material. Table 7 summarizes some examples of fermentation processes producing bioelectricity from wastes. In the last years, lignocellulosic biomasses from agricultural residues (Ren et al. 2007); brewery, synthetic (Venkata Mohan et al. 2008), starch processing (Lu et al. 2009), and dye (Sun et al. 2009) wastewaters; and other wastes have been used for generating electricity by applying the MFCs technology; among them, one of the most suitable in the MFCs system is the wastewater from breweries since it is characterized by low concentrations of inhibitory substances, and its organic matter is a food-derived nature (Feng et al. 2008). Abhilasha and Sharma (2009) compared the electricity production capacity of beer brewery wastewater, sugar industry wastewater, dairy wastewater, municipal wastewater, and paper industry wastewater through MFCs obtaining up to 14.92 mA current in 10 days of operation by using MFC. Recently, it has been demonstrated that using the sequential anode–cathode configuration microbial fuel cells (SACMFC) coupled with photobioreactor, it is possible to improve the treatment of the domestic wastewater to generate electricity. Wastewater was first treated with the SACMFC to remove chemical oxygen demand (COD), phosphorus, and nitrogen and to generate electricity. Then, the treated wastewaters were used to further remove the residual phosphorus and nitrogen by using microalgae (Jiang et al. 2012). Mohan and Chandrasekhar (2011) tried to recover bioelectricity from biodegradable organic matter present in food waste by integrating *solid-state fermentation* with MFC. Experiments demonstrated the possibility of using designed solid-phase microbial fuel cell configurations for generating bioelectricity from solid-state fermentation of composite canteen-based solid food waste. An Integrated Biohydrogen Refinery (IBHR) is described by Redwood et al. (2012). This refinery is aimed at converting biomass to electricity using hydrothermal hydrolysis, extractive biohydrogen fermentation, and photobiological hydrogen fermentation. In this study, an extractive fermentation is applied to waste-derived substrates following hydrothermal pretreatment, achieving 83–99 % biowaste destruction. The selective separation of organic acids from waste-fed fermentations offers appropriate substrate for photofermentative hydrogen production, which enhanced the energy generation up to 11-fold. The MFC represents an important milestone for the energy of the future since all the current methods to generate energy are not sustainable and are based on sources that are not renewable. Despite the last researches on MFCs have resulted in a 10,000-fold increase in the current density obtained from MFCs in small lab-scale system (Rabaey et al. 2004; Venkata Mohan et al. 2007; Schroder 2007; Biffinger et al. 2008; Lovely and Nevin 2008; You et al. 2008), the scale-up is yet a great challenge due the existing limitations like the high cost of the facilities.

Added value products from wastes by microbial solid-state fermentation

Solid-state fermentation (SSF) is carried out in the absence or near-absence of free water (Pandey 2003), and it is characterized by low energy consumption, high volumetric productivity, higher titer of added value products, less waste generation, and low catabolic repression (Hölker et al. 2004). Different kinds of wastes have been successfully used as substrates for microbial solid-state fermentation to produce a variety of products with great economical advantage as reported following. An easy pretreatment of solid wastes, including grinding and material classification in different granulometries to achieve a better material homogenization and to assure less influence of this parameter on the next step, is needed to allow microbial colonization. In this way, SSF represents an attractive alternative to Submerged Fermentation (SmF) process. Table 8 summarizes some examples of solid-state fermentation processes producing added value products from wastes.

Table 7 Examples of fermentation processes producing biopolymers

Biosystem	Waste	Product	Reference
Pseudomonas pseudoflava ATCC 33668	Hemicellulose fraction of poplar wood	Polyhydroxyalkanoate (biopolymer)	Bertrand et al. (1990)
Burkholderia cepacia	Xylose derived from forest biomass	Polyhydroxyalkanoate (biopolymer)	Keenan et al. (2006)
Cordyceps sinensis 16	Molasses	Exo-biopolymer (biopolymer)	Cha et al. (2007)
Microbial community engineering	Glycerol	Polyhydroxyalkanoate (biopolymer)	Moralejo-Gárate et al. (2011)
Bacillus megaterium	Glycerol	Polyhydroxybutyrate (biopolymer)	Naranjo et al. (2013)

Table 8 Examples of solid-state fermentations producing added value products from wastes

Organism	Waste	Product	References
Rhizopus oryzae	Food-processing wastes	Lipases (enzymes)	
Pleurotus florida	Agro wastes	Laccases (enzymes)	Sathishkumar et al. (2010)
Aspergillus carneus M34	Agro wastes	Xylanases (enzymes)	Fang et al. (2010)
Eight *Fusarium culmorum*, 4 *Fusarium solani*, 6 *Fusarium verticillioides*, and 3 *Fusarium equiseti*	Wheat straw, wheat bran, beet pulp, and cotton seed cake	Xylanases (enzymes)	Arabi et al. (2011)
Aspergillus glaucus MN1, *Aspergillus oryzae* MN2, and *Penicillum purpurogenum* MN3	Rice husks, wheat bran, corn cobs, wheat straw, and rice straw	Cellulases and amylases (enzymes)	El-Naggar and El-Hersh (2011)
Pleurotus ostreatus and *Trametes versicolor*	Wastewater	Laccases and xylanases (enzymes)	Iandolo et al. (2011b)
Pleurotus ostreatus and *Trametes versicolor*	Tomato pomace	Laccases and xylanases (enzymes)	Iandolo et al. (2011a)
Aspergillus niveus	Agro wastes	Thermostable polygalacturonase (enzymes)	Maller et al. (2011)
Sewage sludge as source of microorganisms	Vegetable oil-refining wastes	Lipases (enzymes)	Santis-Navarro et al. (2011)
Pleurotus ostreatus	Tomato pomace	Glycosyl-idrolase (enzymes)	Amore et al. (2012)
Pleurotus ostreatus	Sugarcane bagasse	Laccases (enzymes)	Karp et al. (2012)
Grifola frondosa	Oak-sawdust plus corn bran, and oak/corn bran supplemented with coffee spent-ground	Xylanases and cellulases (enzymes)	Montoya et al. (2012)
Aspergillus terreus	Mild alkali-treated rice straw	Cellulases (enzymes)	Narra et al. (2012)
Panus tigrinus	Rice straw, rice husk, and cassava peel	Laccases and lignin peroxidases (enzymes)	Ruqayyah et al. (2013)
Aspergillus niger	Molasses	Citric acid (organic acids)	Garg and Sharma (1992)
Aspergillus niger	Molasses	Citric acid (organic acids)	Pera and Caallieri (1997)
Aspergillus niger	Cheese whey	Citric acid (organic acids)	El-Samragy et al. (1996)
Aspergillus niger	Whey	Citric acid (organic acids)	Hossain et al. (1983)
Aspergillus niger	Whey with sugars and additives	Citric acid (organic acids)	El-Holi and Al-Delaimy (2003)
Aspergillus niger	*Zea* maize	Citric acid (organic acids)	Esuoso et al. (1991)
Aspergillus niger NRRL 599	Orange processing waste	Citric acid (organic acids)	Aravantinos-Zafiris et al. (1994)
Aspergillus niger	Carob pod	Citric acid (organic acids)	Fyllos (1998)
Aspergillus niger GCMC-7	Molasses	Citric acid (organic acids)	Haq et al. (2001)
Aspergillus niger NRRL-567	Apple pomace waste supplemented with rice husk	Citric acid (organic acids)	Dhillon et al. (2011)
Phanerochaete chrysosporium F1767, *Phlebia radiata* 79, and *Ceriporiopsis subvermispora* FP-90031-sp	Wheat straw	Oxalic acid (organic acids)	Galkin et al. (1998)
Bacillus sp. strain XZL9	Corncob molasses	L-lactic (organic acids)	Wang et al. (2010)
Lentinula edodes	Agro-industrial and lignocellulosic wastes	Lentinan (pharmaceutical products)	Israilides and Philippoussis (2003); Philippoussis et al. (2007); Zhang et al. (2007)
Aspergillus niger CGMCC0774 and *Pycnoporus cinnabarinus* CGMCC1115	Waste residue of rice bran oil	Vanillin (pharmaceutical products)	Zheng et al. (2007)
Lentinus spp., *Lentinula* spp., *Leonotis* spp., *Pleurotus* spp., *Agaricus* spp., *Agrocybe* spp., *Volvariella* spp., and *Grifola* spp.	Agricultural wastes (tomato skins, corn stalk husks and cobs, wheat straw, olive mill solid waste, madake bamboo, etc.)	Animal feed (food and feed)	Israilides et al. (1981)
Pleurotus spp.	Agro-industrial wastes	Animal feed (food and feed)	Alborés et al. (2006)
Pleurotus sajor-caju	Rice and wheat straw	Animal feed (food and feed)	Bisaria et al. (1997)
Pleurotus ostreatus and *Trametes versicolor*	Wheat straw	Animal feed (food and feed)	Shrivastava et al. (2011)

Table 8 (continued)

Organism	Waste	Product	References
Saccharomyces cerevisiae	Cucumber and orange peels	Animal feed and human food (food and feed)	Mondal et al. (2012)

Enzymes

Production of industrially relevant enzymes represents a field of increasing interest of biotechnology sciences. The global market for industrial enzymes was estimated at $3.3 billion in 2010, and it is expected to reach $4.4 billion by 2015, with a compound annual growth rate (CAGR) of 6 % over the 5-year forecast period. Oxidative enzymes, i.e., laccases (Piscitelli et al. 2011), lignin peroxidases, and manganese peroxidases (Hofrichter et al. 2010), and hydrolytic enzymes, i.e., xylanases (Bhat 2000), cellulases (Phitsuwan et al. 2013), proteases (Anwar and Saleemuddin 1998), and lipases (Nagarajan 2012), attract much interest because of their applications in different industrial fields, such as pulp and paper industry, food industry, animal feed industry, textile industry, detergent, pharmaceutical, leather, and silk industries. Moreover, oxidative enzymes represent useful biocatalysts for bioremediation processes, being able of degrading huge variety of toxic and recalcitrant compounds. Large amounts of these enzymes are needed to make possible their application at industrial scale, thus considerably increasing costs of industrial processes. Reducing the costs of enzymes is a key research priority for reducing the costs of several industrial processes, especially those that historically have caused adverse environmental impacts and, consequently, may have incentive to use more environmentally benign processes like those based on enzymes. From this point of view, SSF holds tremendous potential for the production of industrial enzymes because of its economic advantages (Pandey et al. 2000). Singhania et al. (2010), for example, highlighted the economic advantages of SSF processes comparing cellulase production in SmF (submerged fermentation) and SSF processes, reporting about tenfold reduction in production cost when cellulases were produced in SSF when compared to SmF.

Much work has been carried out on the production of enzymes of industrial importance by SSF processes with different microorganisms on agro-industrial residues. SSF of basidiomycetes fungi represents the best system for producing fungal enzymes because of the similarity between their natural environment and the conditions in which SSF processes are carried out (Viniegra-González et al. 2003).

Several examples of production of enzymes by SSF of fungi on wastes were so far reported, i.e., by Couto (2008), López et al. (2010), Sathishkumar et al. (2010), Fang et al. (2010), Arabi et al. (2011), El-Naggar and El-Hersh (2011), Iandolo et al. (2011a, b), Maller et al. (2011), Santis-Navarro et al. (2011), Amore et al. (2012), Karp et al. (2012), Montoya et al. (2012), Narra et al. (2012), and Ruqayyah et al. (2013).

One of the main bottlenecks of SSF is the complicated downstream process of the enzymes of interest, due to the presence of particulates and to the complex composition of the substrates. Therefore, more research is needed to improve downstream processing of the added value products produced by SSF.

Organic acids

Citric acid is largely employed for commercial use; indeed, approximately 75 % of it is used in food and 12 % in pharmaceutical industries. There are several waste materials being used for the production of citric acid in the world, such as molasses (Garg and Sharma 1992; Pera and Caallieri 1997; Fedoseev 1970; Jianlong et al. 2000), whey (El-Samragy et al. 1996; Hossain et al. 1983; El-Holi and Al-Delaimy 2003), *Zea* maize (Esuoso et al. 1991), orange processing waste (Aravantinos-Zafiris et al. 1994), and carob pod (Fyllos 1998). It is produced mainly by submerged fermentation using *A. niger* or *Candida* sp. from different sources of carbohydrates, such as molasses and starch-based media (Haq et al. 2001). A solid-state fermentation for citric acid production from pineapple waste by *A. niger* KS-7 was developed by Kareem et al. (2010). It was found that pineapple waste with 15 % (*w*/*v*) sucrose and ammonium nitrate (0.25 %, *w*/*v*) gave the optimum citric acid secretion (60.61 g/kg) in the presence of methanol (2 %, *v*/*v*) when fermented for 5 days at 30 °C with the initial moisture content of 65 %.

The effect of two crucial process parameters for solid-state citric acid fermentation by *A. niger* NRRL-567 using apple pomace waste supplemented with rice husk was thoroughly investigated through response surface methodology by Dhillon et al. (2011). Moisture and methanol had significant positive effect on citric acid production. Higher values of citric acid on apple pomace by *A. niger* (342.41 and 248.42 g/kg dry substrate) were obtained with 75 % (*v*/*w*) moisture along with two inducers [3 % (*v*/*w*) methanol and 3 % (*v*/*w*) ethanol] with fermentation efficiency of 93.90 % and 66.42 %, respectively, depending upon the total carbon utilized after 144 h of incubation period. These results show

that apple pomace is the promising substrate for the hyper production of citric acid through solid-state tray fermentation, which is an economical technique and does not require any sophisticated instrumentation.

Production of oxalic acid by different white-rot fungi during solid-state fermentation has been detected by Galkin et al. (1998). They worked with 15 different fungal strains, and they found an oxalic acid accumulation by *Phanerochaete chrysosporium* F1767 (ATCC 24725), *Phlebia radiata* 79 (ATCC 64658), and *Ceriporiopsis subvermispora* FP-90031-sp; the highest level of oxalate was detected in the cultures of *C. subvermispora* on the 12th day of growth (2.6 mM). In all three fungi, the accumulation of oxalate occurred during stationary growth phase and peaked approximately between the first and second growth weeks, then decreasing quite starkly.

Wang et al. (2010) have provided encouraging data of producing L-lactic acid from lignocellulosic resource. They obtained a high concentration of L-lactic acid (74.7 g/L) from corncob molasses (initial total sugars of 91.4 g/L, especially xylose) in fed-batch fermentation, utilizing *Bacillus* sp. strain XZL9.

Pharmaceutical products

The fungal cultivation on lignocellulosic wastes allows producing a lot of high added value products with pharmaceutical applications, such as hormones, vitamins, and enzymes. In particular, the cultivation of *Lentinula edodes* has been reported for the bioconversion of many types of low-value agro-industrial and lignocellulosic wastes into higher value products such as anti-tumor, anti-inflammatory, anti-virus, anti-bacterial polysaccharides and compounds with immunomodulating properties, as well as active substances that lower cholesterol (Israilides and Philippoussis 2003; Philippoussis et al. 2007; Zhang et al. 2007). Among these, lentinan, a polysaccharide obtained from the fruiting body or the mycelium of the mushroom *L. edodes*, has found the major industrial applications in the pharmaceutical industry. It can be used as an adjuvant in some cancer therapies (Borchers et al. 1999), as an anticlotting agent (Subbarao et al. 1979), and also in patients with bacterial and parasitic infections (Jones 1988). Recently, industrial applications of the underutilized by-products, like bioactive peptides, collagen, gelatin, and oligosaccharides, generated from the seafood processing plants were discovered (Senevirathne and Kim 2012). Bioactive peptides derived from fish by-products have shown various biological activities including antihypertensive and antioxidant activities; collagen and gelatin are currently used in different fields including food, cosmetic, and biomedical industries (Senevirathne and Kim 2012).

Vanillin is one of the products which have attracted great interest (Walton et al. 2003), since it is used in various fields including the chemical and pharmaceutical industries for the production of herbicides, anti-foaming agents or drugs such as papaverine, -dopa, and the anti-microbial agent, trimethoprim. Zheng et al. (2007) developed a new technology to transform ferulic acid, which was from waste residue of rice bran oil, into vanillin using a combination of fungal strains *A. niger* CGMCC0774 and *Pycnoporus cinnabarinus* CGMCC1115. The highest yield reached 2.2 g/L of vanillic acid by *A. niger* CGMCC0774 in a 25-L fermenter when concentration of ferulic acid was 4 g/L. The filtrate of *A. niger* CGMCC0774 culture was concentrated, and vanillic acid in the filtrate was bio-converted into vanillin by *P. cinnabarinus* CGMCC1115. The yield of vanillin reached 2.8 g/L when 5 g/L of glucose and 25 g of HZ802 resin were supplemented in the bioconversion medium.

Food and feed

Bioconversion of wastes through mushroom cultivation and single cell protein (SCP) represents an innovative and alternative system that offers the potential to obtain protein-rich attractive food, supplementing the conventional protein source. The utilization of agricultural wastes, such as tomato skins, corn stalk husks, and cobs (Israilides and Henderson 1981), allows obtaining high value-added products, which are being utilized as animal feed. Edible mushrooms such as *Lentinus* spp., *Lentinula* spp., *Leonotis* spp., *Pleurotus* spp., *Agaricus* spp., *Agrocybe* spp., *Volvariella* spp., and *Grifola* spp., able to grow on a wide range of biomasses, including lignocellulosic and agro-industrial wastes, have been largely studied for their ability to ferment the different crop residues (wheat straw, olive mill solid waste, madake bamboo, etc.) to produce improved animal feed. The effects of fungi on lignocellulosic wastes are focused on two points: they increase the digestibility of the lignocellulosic material through their enzymatic activities, but they are also able to use the substrates to benefit and increase the protein content. Moreover, fungi-treated lignocellulosic feeding carries no risk for animals (Howard et al. 2003), so they provide an economically acceptable alternative for the production of food of superior taste and quality which does not need isolation and purification (Israilides and Philippoussis 2003; Philippoussis et al. 2007). Alborés et al. (2006) showed that, after 14 days of SSF, the edible mushroom *Pleurotus* spp. allows to obtain the bioconversion of the agro-industrial wastes with so as to increase nutritional values and digestibility for its use as animal feed. In another study in which rice straw and wheat straw were used as substrates, *Pleurotus sajor-caju* used as fungi, the protein content of rice and wheat straw increased from 2.87 % to 6.3 % and from 3.1 % to 7.5 % at the end of a 20-day incubation (Bisaria et al. 1997). In order to improve the digestibility and nutrient availability in rumen, wheat straw

was subjected to solid-state fermentation (SSF) with white-rot fungi *Pleurotus ostreatus* and *Trametes versicolor* by Shrivastava et al. (2011). The fungal treatment after 30 days led to the significant decrease in acid detergent fiber (ADF), neutral detergent fiber (NDF), hemicellulose, lignin, and cellulose to the extent of 35.00 %, 38.88 %, 45.00 %, 37.48 %, and 37.86 %, respectively, in *P. ostreatus* fermented straw, while 30.04 %, 33.85 %, 39.90 %, 31.29 %, and 34.00 %, respectively, in *T. versicolor* fermented straw. However, maximum efficiency of fermentation in terms of low carbohydrate consumption per unit of lignin degradation favoring cattle feed production was observed for *P. ostreatus* on the tenth day (17.12 %) as compared with *T. versicolor* on the 30th day (16.91 %). The myco-straw was found to contain significantly high crude protein (4.77 % *T. versicolor*, 5.08 % *P. ostreatus*) as compared to control straw (3.37 %).

Moreover, another potential way that allows the conversion of low-quality biomass into an improved animal feed and human food is based on the utilization of protein extracted from cultivated microbial biomass (SCP). The production of SCP using *S. cerevisiae* by submerged fermentation on cucumber and orange peels was evaluated. Cucumber peel generates higher amount of protein followed by that of orange with 53.4 % and 30.5 % crude protein, respectively, per 100 g of substrate used. It was demonstrated that the SCP production by yeast depends on the growth substrates or media composition since that the percentage of SCP was much lower (17.47 %) when the growth medium contains inorganic nitrogen sources but devoid of glucose, while the addition of glucose enhanced the protein content (60.31 %; Mondal et al. 2012).

Conclusions

A wide range of high added value products such as enzymes, biofuels, organic acids, biopolymers, bioelectricity, and molecules for food and pharmaceutical industries can be obtained upgrading solid wastes. The reported examples of the microbial processes so far exploited for upgrading wastes highlight the variety of transformable wastes. The adopted wastes have to be chosen on a local basis taking the amounts and the current disposal systems into account. A large spectrum of food and fruit processing wastes should be used to ensure the continuous feeding of the production system considering the different seasonal availability. This variability and the heterogeneous character of the other most abundant type of solid wastes (MSW) make the need for an adaptation of raw material composition or the development of a more flexible conversion system.

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References

Abdel-Rahman MA, Tashiro Y, Zendo T, Hanada K, Shibata K, Sonomoto K (2011) Efficient homofermentative L-(+)-lactic acid production from xylose by a novel lactic acid bacterium, *Enterococcus mundtii* QU 25. Appl Environ Microbiol 77(5):1892–1895

Abhilasha SM, Sharma VN (2009) Bioelectricity production from various wastewaters through microbial fuel cell technology. J Biochem Technol 2(1):133–137

Adler PB, Seabloom EW, Borer ET, Hillebrand H, Hautier Y, Hector A, Harpole WS, O'Halloran LR, Grace JB, Anderson TM, Bakker JD, Biederman LA, Brown CS, Buckley YM, Calabrese LB, Chu CJ, Cleland EE, Collins SL, Cottingham KL, Crawley MJ, Damschen EI, Davies KF, DeCrappeo NM, Fay PA, Firn J, Frater P, Gasarch EI, Gruner DS, Hagenah N, Hille Ris Lambers J, Humphries H, Jin VL, Kay AD, Kirkman KP, Klein JA, Knops JMH, La Pierre KJ, Lambrinos JG, Li W, MacDougall AS, McCulley RL, Melbourne BA, Mitchell CE, Moore JL, Morgan JW, Mortensen B, Orrock JL, Prober SM, Pyke DA, Risch AC, Schuetz M, Smith MD, Stevens CJ, Sullivan LL, Wang G, Wragg PD, Wright JP, Yang LH (2011) Productivity is a poor predictor of plant species richness. Science 333(6050):1750–1753

Adsul MG, Varma AJ, Gokhale DV (2007) Lactic acid production from waste sugarcane bagasse derived cellulose. Green Chem 9:58–62

Akin-Osanaiye BC, Nzelibe HC, Agbaji AS (2008) Ethanol production from *Carica papaya* (pawpaw) fruit waste. Asian J Biochem 3(3):188–193

Akköse S, Gündüz U, Yücel M, Eroglu I (2009) Effects of ammonium ion, acetate and aerobic conditions on hydrogen production and expression levels of nitrogenase genes in *Rhodobacter sphaeroides* O.U.001. Int J Hydrog Energy 34(21):8818–8827

Alborés SA, Pianzzolla MJ, Soubes M, Cerdeiras MP (2006) Biodegradation of agroindustrial wastes by *Pleurotus* spp for its use as ruminant feed. Biotechnol Environ 9(3):215–220

Alva Munoz LE, Riley MR (2008) Utilization of cellulosic waste from tequila bagasse and production of polyhydroxyalkanoate (PHA) bioplastics by *Saccharophagus degradans*. Biotechnol Bioeng 100(5):882–888

Álvarez JA, Otero L, Lema JM (2010) A methodology for optimizing feed composition for anaerobic co-digestion of agro-industrial wastes. Bioresour Technol 101:1153–1158

Amore A, Faraco V (2011) Potential of fungi as category I Consolidated BioProcessing organisms for cellulosic ethanol production. Renew Sust En Rev 16:3286–3301

Amore A, Amoresano A, Birolo L, Henrissat B, Leo G, Palmese A, Faraco V (2012). A family GH51 α-L-arabinofuranosidase from Pleurotus ostreatus: identification, recombinant expression and characterization. Appl Microbiol Biotechnol 94(4):995–1006

Anwar A, Saleemuddin M (1998) Alkaline proteases: a review. Bioresour Technol 64:175–183

Arabi MI, Bakri Y, Jawhar M (2011) Extracellular xylanase production by *Fusarium* species in solid state fermentation. Pol J Microbiol 60(3):209–212

Aravantinos-Zafiris G, Tzia G, Oreopaulai V, Thomopoulos CD (1994) Fermentation of orange processing wastes for citric acid production. J Sci Food Agric 64:117–120

Argun H, Kargi F (2010) Bio-hydrogen production from ground wheat starch by continuous combined fermentation using annular-hybrid bioreactor. Int J Hydrog Energy 35(12):6170–6178

Asli MS (2010) A study on some efficient parameters in batch fermentation of ethanol using *Saccharomyces cerevesiae* SC1 extracted from fermented Siahe Sardasht pomace. Afr J Biotechnol 9(20):2906–2912

Balan V, Bals B, Chundawat SP, Marshall D, Dale BE (2009) Lignocellulosic biomass pretreatment using AFEX. Methods Mol Biol 581:61–77

Basak N, Das D (2006) The prospect of purple non-sulfur (PNS) photosynthetic bacteria for hydrogen production: the present state of the art. W J Microbiol Biotechnol 23(1):31–42

Bertrand JL, Ramsay BA, Ramsay JA, Chavarie C (1990). Biosynthesis of Poly-beta-Hydroxyalkanoates from Pentoses by *Pseudomonas pseudoflava*. Appl Microbiol Biotechnol 56(10):3133–3138

Bhat MK (2000) Cellulase and their related enzymes in biotechnology. Biotechnol Adv 18:355–383

Bhatia L, Johri S, Ahmad R (2012) An economic and ecological perspective of ethanol production from renewable agro waste: a review. AMB Express 2:65–84

Biffinger JC, Pietron J, Bretschger O, Nadeau LJ, Johnson GR, Williams CC, Nealson KH, Ringeisen BR (2008) The influence of acidity on microbial fuel cells containing *Shewanella oneidensis*. Biosens Bioelectron 24(4):900–915

Bisaria R, Madan M, Vasudevan P (1997) Utilization of agro-industries as animal feed through bioconversion. Bioresour Technol 59:5–8

Borchers AT, Stern JS, Hackma RM, Keen C, Gershwin ME (1999) Mushrooms, tumors and immunity. Proc Soc Exp Biol Med 229(5):393–406

Braber K (1995) Anaerobic digestion of municipal solid waste: a modern waste disposal option on the verge of breakthrough. Bio Bioener 9:365–376

Brown D, Li Y (2013) Solid state anaerobic co-digestion of yard waste and food waste for biogas production. Bioresour Technol 127:275–280

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The carbohydrate-active enzymes database (CAZy): an expert resource for glycogenomics. Nucleic Acids Res 37:D233–D238

Cha SH, Limb JS, Yoonc CS, Kohd JH, Change HI, Kim SW (2007) Production of mycelia and exo-biopolymer from molasses by *Cordyceps sinensis* 16 in submerged culture. Bioresour Technol 98(1):165–168

Chaudhary N, Sharma CB (2005) Production of citric acid and single cell protein from agrowaste. Nat Acad Sci Lett 28(5/6):189–193

Chen C, Yeh K, Lo Y, Wang H, Chang J (2010) Engineering strategies for the enhanced photo-H2 production using effluents of dark fermentation processes as substrate. Int J Hydrog Energy 35(24):13356–13364

Cho DH, Lee YJ, Um Y, Sang BI, Kim YH (2009) Detoxification of model phenolic compounds in lignocellulosic hydrolysates with peroxidase for butanol production from *Clostridium beijerinckii*. Appl Microbiol Biotechnol 83:1035–1043

Chundawat S, Beckham G, Himmel M, Dale B (2011) Deconstruction of lignocellulosic biomass to fuels and chemicals. Annu Rev Chem Biomol Eng 2:121–145

Claassen PAM, Budde MAW, López-Contreras AM (2000) Acetone, butanol and ethanol production from domestic organic waste by solventogenic *Clostridia*. J Mol Microbiol Biotechnol 2(1):39–44

Couto SR (2008) Exploitation of biological wastes for the production of value-added products under solid-state fermentation conditions. Biotechnol J 3:859–870

Das D, Veziroğlu TN (2001) Hydrogen production by biological processes: a survey of literature. Int J Hydrog Energy 26:13–28

Datar R, Huang J, Maness PC, Mohagheghi A, Czemik S, Chornet E (2007) Hydrogen production from the fermentation of corn stover biomass pretreated with a steam-explosion process. Int J Hydrog Energy 32:932–939

Dhillon GS, Brar SK, Verma M, Tyagi RD (2011) Enhanced solid-state citric acid bio-production using apple pomace waste through surface response methodology. J Appl Microbiol. doi:10.1111/j.1365-2672.2011.04962.x

Dumbrepatil A, Adsul M, Chaudhari S, Khire J, Gokhale D (2008) Utilization of molasses sugar for lactic acid production by *Lactobacillus delbrueckii* subsp. *delbrueckii* mutant Uc-3 in batch fermentation. Appl Environ Microbiol 74:333–335

Durre P (1998) New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. Appl Microbiol Biotechnol 49:639–648

Elango D, Pulikesi M, Baskaralingam P, Ramamurthi V, Sivanesan S (2007) Production of biogas from municipal solid waste with domestic sewage. J Hazard Mater 141:301–304

El-Holi MA, Al-Delaimy KS (2003) Citric acid production from whey with sugars and additives by *Aspergillus niger*. African J Biotechnol 2(10):356–359

El-Naggar N-A, El-Hersh MS (2011) Organic acids associated with saccharification of cellulosic wastes during solid-state fermentation. J Microbiol 49(1):58–65

El-Samragy YA, Khorshid MA, Foda MI, Shihata AE (1996) Effect of fermentation conditions on the production of citric acid from cheese whey by *Aspergillus niger*. Int J Food Microbiol 29:411–416

Esuoso KO, Oderinde RA, Okogun JI (1991) Citric acid production from imumu *Cyprus esculentus* and maize *Zea*. J Ferment Bioeng 71:200–202

Ezeji TC, Qureshi N, Blaschek HP (2007) Butanol production from agricultural residues: impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation. Biotechnol Bioeng 97(6):1460–1469

Fan JH, Feng WL, Di SM, Yan HR, Jia JJ, Wang ZP (2010) Production of butanol from sugar beet molasses by fed-batch fermentation. J Bioproc Eng 8:6–9

Fang TJ, Liao BC, Lee SC (2010) Enhanced production of xylanase by *Aspergillus carneus* M34 in solid-state fermentation with agricultural waste using statistical approach. N Biotechnol 27(1):25–32

Faraco V, Hadar Y (2011) The potential of lignocellulosic ethanol production in the Mediterranean basin. Renew Sust Energ Rev 15:252–266

Fascetti E, D'Addario E, Todini O, Robertiello A (1998) Photosynthetic hydrogen evolution with volatile organic acids derived from the fermentation of source selected municipal solid wastes. Int J Hydrog Energy 23(9):753–760

Fatehi P (2013) Recent advancements in various steps of ethanol, butanol, and isobutanol productions from woody materials. Biotechnol Prog 29(2):297–310. doi:10.1002/btpr.1688

Fedoseev VF (1970) Effect of copper on the fermentative conversion of molasses to citric acid. Khlebopek Konditer Promst 14:33–35

Feng Y, Wang X, Logan BE, Lee H (2008) Brewery wastewater treatment using air-cathode microbial fuel cells. Appl Microbiol Biotechnol 78:873–880. doi:10.1007/s00253-008-1360-2

Fernandez J, Perez M, Romero LI (2008) Effect of substrate concentration on dry mesophilic anaerobic digestion of organic fraction of municipal solid waste (OFMSW). Biores Technol 99:6075–6080

Fyllos RT (1998) Carob pod, a new substrate for citric acid production by *Aspergillus niger*. Appl Biochem Biotechnol 74:43–53

Galkin S, Vares T, Kalsi M, Hatakka A (1998) Production of organic acids by different white-rot fungi as detected using capillary zone electrophoresis. Biotech Tech 12(4):267–271

Garg K, Sharma CB (1992) Continuous production of citric acid by immobilized whole cells of *Aspergillus niger*. J Gen Appl Microbiol 38:605–615

Givry S, Prevot V, Duchiron F (2008) Lactic acid production from hemicellulosic hydrolyzate by cells of *Lactobacillus bifermentans*

immobilized in Ca-alginate using response surface methodology. World J Microbiol Biotechnol 24:745–752
Gottschalk G (1986) Bacterial metabolism, 2nd edn. Springer, New York
Guo F, Peng ZG, Dai JY, Xiu ZL (2010) Calcined sodium silicate as solid base catalyst for biodiesel production. Fuel Process Technol 91:322–328
Guo F, Fang Z, Zho T (2012) Conversion of fructose and glucose into 5-hydroxymethylfurfural with lignin-derived carbonaceous catalyst under microwave irradiation in dimethyl sulfoxide–ionic liquid mixtures. Bioresour Technol 112:313–318. doi:10.1016/j.biortech.2012.02.108
Guwy AJ, Dinsdale RM, Kim JR, Massanet-Nicolau J, Premier G (2011) Fermentative biohydrogen production systems integration. Bioresour Technol 102:8534–8542
Hahn-Hägerdal B, Galbe M, Gorwa-Grauslund MF, Lidén G, Zacchi G (2006) Bio-ethanol: the fuel of tomorrow from the residues of today. Trends Biotech 24(12):449–556
Haq I, Ali S, Qadeer MA (2001) Fed-batch culture studies during citric acid fermentation by *Aspergillus niger* GCMC-7. Biologia 45:32–37
Hartmann H, Møller HB, Ahring BK (2004) Efficiency of the anaerobic treatment of the organic fraction of municipal solid waste: collection and pretreatment. Waste Manage Res 22:35–41
Henrique M, Baudel HM, Zaror C, de Abreu CAM (2005) Improving the value of sugarcane bagasse wastes via integrated chemical production systems: an environmentally friendly approach. Ind Crops Prod 21(3):309–315
Hipolito CN, Crabbe E, Badillo CM, Zarrabal OC, Morales Mora MA, Pineda Flores G, de A. Hernández Cortazar M, Ishizaki A (2008). Bioconversion of industrial wastewater from palm oil processing to butanol by *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564). J of Clean Product 16:632–638
Hofrichter M, Ullrich R, Pecyna MJ, Liers C, Lundell T (2010) New and classic families of secreted fungal heme peroxidases. Appl Microbiol Biotechnol 87(3):871–897
Hölker U, Höfer M, Lenz J (2004) Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Appl Microbiol Biotechnol 64:175–186
Hossain M, Brooks JD, Maddox IS (1983) Production of citric acid from whey permeate by fermentation using *Aspergillus niger*. J Dairy Sci Technol 18:161–168
Howard RL, Abotsi E, Jansen Van Rensburg EL (2003) Lignocellulose biotechnology: issues of bioconversion and enzyme production. Afr J Biotechnol 2(12):602–619
Hussy I, Hawkes FR, Dinsdale R, Hawkes DL (2003) Continuous fermentative hydrogen production from a wheat starch co-product by mixed microflora. Biotechnol and Bioengin 84(6):619–626
Iandolo D, Amore A, Birolo L, Leo G, Olivieri G, Faraco V (2011a) Fungal solid state fermentation on agro-industrial wastes for acid wastewater decolourization in a continuous flow packed-bed bioreactor. Bioresour Technol 102:7603–7607
Iandolo D, Piscitelli A, Sannia G, Faraco V (2011b) Enzyme production by solid substrate fermentation of *Pleurotus ostreatus* and *Trametes versicolor* on tomato pomace. Appl Biochem Biotechnol 163(1):40–51
Ibrahim MNM, Nadiah MYN, Norliyana MS, Sipaut CS, Shuib S (2008) Separation of vanillin from oil palm empty fruit bunch lignin. Acta hydrochimica et hydrobiologica 36(3):287–291
Igoni AH, Ayotamuno MJ, Eze CL, Ogaji SOT, Probert SD (2008) Designs of anaerobic digesters for producing biogas from municipal solid-waste. Appl Ener 85:430–438
Israilides C, Henderson R (1981) Cultivation of *Lentinus edodes* using rye grass straw as a substrate. Agric Res 5:123–128
Israilides C, Philippoussis A (2003) Bio-technologies of recycling agroindustrial wastes for the production of commercially important polysaccharides and mushrooms. Biotechnol Gen Eng Rev 20:247–259
Iyer PV, Thomas S, Lee YY (2000) High-yield fermentation of pentoses into lactic acid. Appl Biochem Biotechnol 84–86:665–667
Jain A, Morlok CK, Henson JM (2013) Comparison of solid-state and submerged-state fermentation for the bioprocessing of switchgrass to ethanol and acetate by *Clostridium phytofermentans*. Appl Microbiol Biotechnol 97(2):905–917
Jiang H, Luo S, Shi X, Dai M, Guo R (2012) A novel microbial fuel cell and photobioreactor system for continuous domestic wastewater treatment and bioelectricity generation. Biotechnol Lett 34:1269–1274. doi:10.1007/s10529-012-0899-2
Jianlong W, Hangchang S, Yi Q (2000) Wastewater treatment in a hybrid biological reactor (HBR): effect of organic rates. Process Biochem 36:279–303
Jin F, Zheng J, Enomoto H, Moriya T, Sato N, Higashijima H (2002) Hydrothermal process for increasing acetic acid yield from lignocellulosic wastes. Chem Lett 31(5):504
Jin F, Cao J, Zhou Z, Moriya T, Enomoto H (2004) Effect of lignin on acetic acid production in wet oxidation of lignocellulosic wastes. Chem Lett 33:910
Jin F, Zhou Z, Kishita A, Enomoto H (2006) Hydrothermal conversion of biomass into acetic acid. J Mater Sci 41(5):1495–1500
John RP, Nampoothiri KM, Pandey A (2006) Simultaneous saccharification and fermentation of cassava bagasse for L-(+)-lactic acid production using *Lactobacilli*. Appl Biochem Biotechnol 134:263–272
Jones K (1988) Shiitake: a major medicinal mushroom. Altern Complement Ther 4:53–59
Joshi SS, Dhopeshwarkar R, Jadav U, Jadav R, D'souza L, Jayaprakash D (2001) Continuous ethanol production by fermentation of waste banana peels using flocculating yeast. Indian J Chem Tech 8:153–159
Kamei I, Hirota Y, Meguro S (2012) Integrated delignification and simultaneous saccharification and fermentation of hard wood by a white-rot fungus, *Phlebia* sp. MG-60. Bioresour Technol 126:137–141
Kanai T, Imanaka H, Nakajima A, Uwamori K, Omori Y, Fukui T, Atomi H, Imanaka T (2005) Continuous hydrogen production by the hyperthermophilic archaeon, *Thermococcus kodakaraensis* KOD1. J Biotechnol 116:271–282
Kareem SO, Akpan I, Alebiowu OO (2010) Production of citric acid by *Aspergillus niger* using pineapple waste. Mal J Microbiol 6(2):161–165
Karp SG, Faraco V, Amore A, Birolo L, Giangrande C, Soccol VT, Pandey A, Soccol CR (2012) Characterization of laccase isoforms produced by *Pleurotus ostreatus* in solid state fermentation of sugarcane bagasse. Bioresour Technol 114:735–739
Keenan TM, Nakas JP, Tanenbaum SW (2006). Polyhydroxyalkanoate copolymers from forest biomass. J Ind Microbiol Biotechnol 33:616–626. doi:10.1007/s10295-006-0131-2
Kiely G, Tayfur G, Dolan C, Tanji K (1997) Physical and mathematical modelling of anaerobic digestion of organic wastes. Water Res 31:534
Kim DH, Han SK, Kim SH, Shin HS (2006) Effect of gas sparging on continuous fermentative hydrogen production. Int J Hydrog Energy 31(15):2158–2169
Koller M, Salerno A, Dias M, Reiterer A, Braunegg G (2010) Modern biotechnological polymer synthesis: a review. Food Technol Biotechnol 48(3):255–269
Koller M, Salerno A, Muhr A, Reiterer A, Chiellini E, Casella S, Horvat P, Braunegg G (2012) Whey lactose as a raw material for microbial production of biodegradable polyesters. In: Saleh HE-D (ed) Polyester. InTech, Manhattan. Available from: http://www.intechopen.com/books/polyester/whey-lactose-as-a-raw-material-for-microbial-production-of-biodegradable-polyesters. ISBN:978-953-51-0770-5. doi:10.5772/48737
Kucukasik F, Yenigun O, Oner ET (2009) Molasses as fermentation substrate for microbial biopolymer production. New Biotechnol 25:S250

Laufenberg G, Kunz B, Nystroem M (2003) Transformation of vegetable waste into value added products: (A) the upgrading concept; (B) practical implementations. Bioresour Technol 87:167–198

Leaf TA, Srienc F (1998) Metabolic modeling of polyhydroxybutyrate biosynthesis. Biotechnol Bioeng 57(5):557–570

Lee TM, Ayaaki I, Sadazo Y, Kensuke F (1995) Production of acetone, butanol and ethanol from palm oil waste by *Clostridium saccharoperbutylacetonicum* N1-4. Biotechnol Lett 17:649–654

Li A (2008) Municipal solid waste used as bioethanol sources and its related environmental impacts. Int J Soil Sediment Water 1(1):1–5

Li A, Antizar-Ladislao B, Khraisheh M (2007) Bioconversion of municipal solid waste to glucose for bio-ethanol production. Bioproc Biosyst Eng 30(3):189–196

Lim SJ, Kim BJ, Jeong CM, Choi J, Ahn YH, Chang HN (2008) Anaerobic organic acid production of food waste in once-a-day feeding and drawing-off bioreactor. Bioresour Technol 99(16):7866–7874

Liu Y, Yu P, Song X, Qu Y (2008) Hydrogen production from cellulose by coculture of *Clostridium thermocellum* JN4 and *Thermoanaerobacterium thermosaccharolyticum* GD17. Int J Hydrog Energy 33:2927–2933

Liu ZY, Ying Y, Li FL, Ma CQ, Xu P (2010) Butanol production by *Clostridium beijerinckii* ATCC 55025 from wheat bran. J Ind Microbiol Biotechnol 37:495–501

Lo YC, Chen CY, Lee CM, Chang JS (2010a) Combining dark-photo fermentation and microalgae photosynthetic processes for high-yield and CO_2-free biohydrogen production. Int J Hydrog Energy 35:10944–10953

Lo YC, Lu WC, Chen CY, Chang JS (2010b) Dark fermentative hydrogen production from enzymatic hydrolysate of xylan and pretreated rice straw by *Clostridium butyricum* CGS5. Bioresour Technol 101:5885–5891

Lopez TM, Espinosa LMC (2008) Effect of alkaline pretreatment on anaerobic digestion of solid wastes. Waste Manag 28:2229–2234

López E, Deive FJ, Longo MA, Sanromán MA (2010) Strategies for utilisation of food-processing wastes to produce lipases in solid-state cultures of *Rhizopus oryzae*. Bioproc Biosyst Eng 33(8):929–935

Lovely DR, Nevin KP (2008) Electricity production with electricigens. In: Wall JD et al (eds) Bioenergy. ASM, Washington, pp 295–306

Lu N, Zhou SG, Zhuang L, Zhnag JT, Ni JR (2009) Electricity generation from starch processing wastewater using microbial fuel cell technology. Biochem Eng J 43:246–251

Lui H, Ramnarayanan R, Logan BE (2004) Production of electricity during wastewater treatment using a single chamber microbial fuel cell. Env Sc Tech 38:2281–2285

Madihah MS, Ariff AB, Sahaid KM, Suraini AA, Karim MIA (2001) Anaerobic fermentation of gelatinized sago starch-derived sugars to acetone-butanol-ethanol solvent by *Clostridium acetobutylicum*. W J Microbiol Biotechnol 17:567–576

Magnusson L, Islam R, Sparling R, Levin D, Cicek N (2008) Direct hydrogen production from cellulosic waste materials with a single-step dark fermentation process. Int J Hydrog Energy 33:5398–5403

Maller A, Damásio AR, da Silva TM, Jorge JA, Terenzi HF, Polizeli Mde L (2011) Biotechnological potential of agro-industrial wastes as a carbon source to thermostable polygalacturonase production in *Aspergillus niveus*. Enzyme Res 2011:289206

Mondal AK, Sengupta S, Bhowal J, Bhattacharya DK (2012) Utilization of fruit wastes in producing single cell protein. Int J Sci Environ Technol 1(5):430–438

Monlau F, Steyer JP, Sambusiti C, Carrere H, Barakat A, Mei Guo X, Latrille E, Trably E (2012) Predictive models of biohydrogen and biomethane production based on the compositional and structural features of lignocellulosic materials. Environ Sci Technol 46(21):12217–12225

Montoya S, Orrego CE, Levin L (2012) Growth, fruiting and lignocellulolytic enzyme production by the edible mushroom *Grifola frondosa* (maitake). World J Microbiol Biotechnol 28(4):1533–1541

Moralejo-Gárate H, Mar'atusalihat E, Kleerebezem R, van Loosdrecht MC (2011) Microbial community engineering for biopolymer production from glycerol. Appl Microbiol Biotechnol 92(3):631–639

Mshandete AM, Björnsson L, Kivaisi AK, Rubindamayugi MST, Mattiasson B (2008) Effect of aerobic pre-treatment on production of hydrolases and volatile fatty acids during anaerobic digestion of solid sisal leaf decortications residues. Afric J Biochem Res 2(5):111–119

Mtui GYS (2009). Recent advances in pretreatment of lignocellulosic wastes and production of value added products. Afr J Biotechnol 8(8):1398–1415

Muruke MHS, Hosea KM, Palangyo A, Heijthuijsen JHFG (2006) Production of lactic acid from waste sisal stems using a *Lactobacillus* isolate. Discov Innov 18(1):1–5

Mutreja R, Das D, Goyal D, Goyal A (2011) Bioconversion of agricultural waste to ethanol by SSF using recombinant cellulase from *Clostridium thermocellum*. Enz Res 2011:340279 (6 p)

Muttara S, Nirmala DJ (1982) Production of alcohol and acetic acid from pineapple waste. J Wat Qual Bul 7(2):76–82

Nagarajan S (2012) New tools for exploring "old friends-microbial lipases". Appl Biochem Biotechnol 168(5):1163–1196

Naranjo JM, Posada JA, Higuita JC, Cardona CA (2013) Valorization of glycerol through the production of biopolymers: the PHB case using *Bacillus megaterium*. Biores Technol 133: 38–44

Narra M, Dixit G, Divecha J, Madamwar D, Shah AR (2012) Production of cellulases by solid state fermentation with *Aspergillus terreus* and enzymatic hydrolysis of mild alkali-treated rice straw. Bioresour Technol 121:355–361

Ni Y, Wang Y, Sun Z (2012) Butanol production from cane molasses by *Clostridium saccharobutylicum* DSM 13864: batch and semicontinuous fermentation. Appl Biochem Biotechnol 166: 1896–1907. doi:10.1007/s12010-012-9614-y

Octave S, Thomas D (2009) Biorefinery: toward an industrial metabolism. Biochimie 91:659–664

Ohkouchi Y, Inoue Y (2007) Impact of chemical components of organic wastes on L(+)-lactic acid production. Bioresour Technol 98(3):546–553

Ojumu TV, Yu J, Solomon BO (2004) Production of polyhydroxyalkanoates, a bacterial biodegradable polymer. African J Biotechnol 3(1):18–24

Olson ES (2001) Conversion of lignocellulosic material to chemicals and fuels. Report No. FC26-98FT40320-19. National Energy Technology Lab, Pittsburgh

Olson DG, McBride JE, Shaw AJ, Lynd Lee R (2011) Recent progress in consolidated bioprocessing. Curr Op Biotech 23:1–10

Özgür E, Afsar N, de Vrije T, Yücel M, Gündüz U, Claassen PAM (2010) Potential use of thermophilic dark fermentation effluents in photofermentative hydrogen production by *Rhodobacter capsulatus*. Inci Eroglu J Clean Prod 18:S23–S28

Pandey A (2003) Solid-state fermentation. Biochem Eng J 13:81–84

Pandey A, Soccol CR, Mitchell D (2000). New developments in solid state fermentation Process Biochem. 35:1153–1169

Parekh M and Blaschek HP (1999). Butanol production by hypersolvent-producing mutant Clostridium beijerinckii BA101 in corn steep water medium containing maltodextrin. Biotechnol Letters 21:45–48

Passoth V, Tabassum MR, Nair HA, Olstorpe M, Tiukova I, Ståhlberg J (2013) Enhanced ethanol production from wheat straw by integrated storage and pretreatment (ISP). Enzyme Microb Technol 52(2):105–110

Pera LM, Caallieri DA (1997) Influence of calcium on fungal growth, hyphal morphology and citric acid production in *Aspergillus niger*. J Technol 42:551–556

Pfromm PH, Amanor-Boadub V, Nelsonc R, Vadlanid P, Madl R (2010) Bio-butanol vs. bio-ethanol: a technical and economic assessment for corn and switchgrass fermented by yeast or *Clostridium acetobutylicum*. Biomass Bioenergy 34:515–524

Philippoussis A, Diamantopoulou P, Israilides C (2007) Productivity of agricultural residues used for the cultivation of the medicinal fungus *Lentinula edodes*. Int Biodeterior Biodegrad 59(3):216–219

Phitsuwan P, Laohakunjit N, Kerdchoechuen O, Kyu KL, Ratanakhanokchai K (2013) Present and potential applications of cellulases in agriculture, biotechnology, and bioenergy. Folia Microbiol (Praha) 58(2):163–176

Piscitelli A, Del Vecchio C, Faraco V, Giardina P, Macellaro G, Miele A, Pezzella C, Sannia G (2011) Fungal laccases: versatile tools for lignocellulose transformation. CR Biologies 334(11):789–794

Qureshi N, Li XL, Hughes S, Saha BC, Cotta MA (2006) Butanol production from corn fiber xylan using *Clostridium acetobutylicum*. Biotechnol Prog 22:673–680

Qureshi N, Saha BC, Dien B, Hector RE, Cotta MA (2010) Production of butanol (a biofuel) from agricultural residues: part I—use of barley straw hydrolysate. Biomass Bioenergy 34:559–565

Rabaey K, Verstraete W (2005) Microbial fuel cells: novel biotechnology for energy generation. Trends Biotechnol 23:291–298

Rabaey K, Boon N, Siciliano SD, Verhaege M, Verstraete W (2004) Biofuel cells select for microbial consortia that self-mediate electron transfer. Appl Environ Microbiol 70:5373–5382

Raganati F, Olivieri G, Procentese A, Russo ME, Salatino P, Marzocchella A (2013) Butanol production by bioconversion of cheese whey in a continuous packed bed reactor. Bioresour Technol 138:259–265

Raikar RV (2012) Enhanced production of ethanol from grape waste. Int J Env Sci 3:2

Rao M, Singh S (2004) Bioenergy conversion studies of organic fraction of MSW: kinetic studies and gas yield-organic loading relationships for process optimisation. Biores Technol 95:173–185

Reddy V, Reddy V (2007) Production of ethanol from mango (*Mangifera indica* L.) fruit juice fermentation. Res J Microbiol 2(10):763–769

Redwood MD, Orozco RL, Majewski AJ, Macaskie LE (2012) An integrated biohydrogen refinery: synergy of photofermentation, extractive fermentation and hydrothermal hydrolysis of food wastes. Bioresour Technol 119:384–392. doi:10.1016/j.biortech.2012.05.040

Ren Z, Ward TE, Regan JM (2007) Electricity production from cellulose in a microbial fuel cell using a defined binary culture. Environ Sci Technol 41:4781–4786

Ruqayyah TI, Jamal P, Alam MZ, Mirghani ME (2013) Biodegradation potential and ligninolytic enzyme activity of two locally isolated *Panus tigrinus* strains on selected agro-industrial wastes. J Environ Manage 118C:115–121

Santis-Navarro A, Gea T, Barrena R, Sánchez A (2011) Production of lipases by solid state fermentation using vegetable oil-refining wastes. Bioresour Technol 102(21):10080–10084

Sasikala K, Ramana CV (1991) Photoproduction of hydrogen from waste water of a lactic acid fermentation plant by a purple non-sulfur photosynthetic bacterium, *Rhodobacter sphaeroides* O.U.001. Indian J Exp Biol 29:74–75

Sathishkumar P, Murugesan K, Palvannan T (2010) Production of laccase from *Pleurotus florida* using agro-wastes and efficient decolorization of Reactive blue 198. J Basic Microbiol 50(4):360–367

Schink B (1997) Energetics of syntrophic cooperation in methanogenic degradation. Microbiol Mol Biol Rev 61:262–280

Schroder U (2007) Anodic electron transfer mechanisms in microbial fuel cells and their energy efficiency. Phys Chem Chem Phys 9:2619–2629

Senevirathne M, Kim SK (2012) Utilization of seafood processing by-products: medicinal applications. Adv Food Nutr Res 65:495–512

Shrivastava B, Khasa YP, Puniya AK, Thakur S, Gupte A, Kuhad RC (2011) White-rot fungal conversion of wheat straw to energy rich cattle feed. Biodegradation 22:823–831

Singh A, Bishnoi NR (2012a) Enzymatic hydrolysis optimization of microwave alkali pretreated wheat straw and ethanol production by yeast. Bioresour Technol 108:94–101. doi:10.1016/j.biortech.2011.12.084

Singh A, Bishnoi NR (2012b) Optimization of enzymatic hydrolysis of pretreated rice straw and ethanol production. Appl Microbiol Biotechnol 93(4):1785–1793

Singh A, Jain VK (1995) Batch fermentation of cane molasses for ethanol production by *Zymomonas mobilis*. J Ind Chem Eng 37:80–94

Singhania RR, Sukumaran RK, Patel AK, Larroche C, Pandey A (2010) Advancement and comparative profiles in the production technologies using solid-state and submerged fermentation for microbial cellulases. Enzyme Microbial Technol 46:541–549

Sosnowski P, Wieczorek A, Ledakowicz S (2003) Anaerobic co-digestion of sewage sludge and organic fraction of municipal solid wastes. Adv Environ Res 7:609–616

Stroot PG, McMahon KD, Mackie RI, Raskin L (2001) Anaerobic codigestion of municipal solid waste and biosolids under various mixing conditions—I. Digester performance. Water Res 35:1804–1816

Su H, Cheng J, Zhou J, Song W, Cen K (2009) Improving hydrogen production from cassava starch by combination of dark and photo fermentation. Int J Hydrog Energy 34(4):1780–1786

Subbarao K, Kuchibhota J, Kaccar BV (1979) Pyridoxal-5-phosphate. A new physiological inhibitor of blood coagulation. Biochem Pharmacol 28:531–534

Sudesh K, Iwata T (2008) Sustainability of biobased and biodegradable plastics. Clean-Soil Air Water 36:433–442

Sun J, Hu YY, Bi Z, Cao YQ (2009) Simultaneous decolorization of azo dye and bioelectricity generation using a microfiltration membrane air-cathode single-chamber microbial fuel cell. Biores Technol 100:3185–3192

Sunita M, Mitra CK (1993) Photoproduction of hydrogen by photosynthetic bacteria from sewage and wastewater. J Biosci 18:155–160

Taherzadeh MJ, Keikhosro K (2007) Enzyme-based hydrolysis processes for ethanol from lignocellulosic materials: a review. BioResources 2:707–738

Türkarslan S, Yiğit DÖ, Aslan K, Eroğlu I, Gündüz U (1998) Photobiological hydrogen production by *Rhodobacter sphaeroides* O.U.001 by utilization of waste water from milk industry. In: Zaborsky OR (ed) Biohydrogen. Plenum, New York, pp 151–156

Uyar B, Eroglu I, Yücel M, Gündüz U (2009) Photofermentative hydrogen production from volatile fatty acids present in dark fermentation effluents. Int J Hydrog Energy 34(10):4517–4523

Venkata Mohan S, Chandrasekhar K (2011) Solid phase microbial fuel cell (SMFC) for harnessing bioelectricity from composite food waste fermentation: influence of electrode assembly and buffering capacity. Bioresour Technol 102:7077–7085. doi:10.1016/j.biortech.2011.04.039

Venkata Mohan S, Veer Raghavulu S, Srikanth S, Sarma PN (2007) Bioelectricity production by mediatorless microbial fuel cell under acidophilic condition using wastewater as substrate: influence of substrate loading rate. Curr Sci 92(12):1720–1726

Venkata Mohan S, Saravanan R, Veer Raghavulu S, Mohanakrishna G, Sarma PN (2008) Bioelectricity production from wastewater treatment in dual chambered microbial fuel cell (MFC) using selectively enriched mixed microflora: effect of catholyte. Biores Technol 99:596–603

Viniegra-González G, Favela-Torres E, Aguilar CN, Romero-Gómez S, Díaz-godínez G, Augur C (2003) Advantages of fungal enzyme

production in solid state over liquid fermentation systems. Biochem Eng J 13:157–167

Walton NJ, Mayer MJ, Narbad A (2003) Molecules of interest: vanillin. Phytochemistry 63:505–6515

Wang L, Zhao B, Liu B, Yu B, Ma C, Su F, Hua D, Li Q, Ma Y, Xu P (2010) Efficient production of L-lactic acid from corncob molasses, a waste by-product in xylitol production, by a newly isolated xylose utilizing *Bacillus* sp. strain. Bioresour Technol 101:7908–7915

Warnick TA, Methe BA, Leschine SB (2002) *Clostridium phytofermentans* sp. nov., a cellulolytic mesophile from forest soil. Int J Syst Evol Micr 52:1155–1160

Weber C, Farwick A, Benisch F, Brat D, Dietz H, Subtil T (2010) Trends and challenges in the microbial production of lignocellulosic bioalcohol fuels. Appl Microbiol Biotechnol 87:1303–1315

Wee YJ, Yun JS, Park DH, Ryu HW (2004) Biotechnological production of L-(+)-lactic acid from wood hydrolyzate by batch fermentation of *Enterococcus faecalis*. Biotechnol Lett 26:71–74

Weiland P (2010) Biogas production: current state and perspectives. Appl Microbiol Biotechnol 85:849–860

Yang B, Dai Z, Ding S-Y, Wyman CE (2011) Enzymatic hydrolysis of cellulosic biomass. Biofuels 2(4):421–450

Yetis M, Gündüz U, Eroğlu I, Yücel M, Türker L (2000) Photoproduction of hydrogen from sugar refinery wastewater by *Rhodobacter sphaeroides* O.U.001. Int J Hydrog Energy 25:1035–1041

You S, Zhao Q, Zhang J, Liu H, Jiang J, Zhao S (2008) Increased sustainable electricity generation in up-flow air-cathode microbial fuel cells. Biosens Bioelectron 23:1157–1160

Yue PL, Lowther K (1986) Enzymatic oxidation of C1 compounds in a biochemical fuel cell. Chem Eng J 33:B69–B77

Zaldivar J, Nielsen J, Olsson L (2001) Fuel ethanol production from lignocellulose: a challenge for metabolic engineering and process integration. Appl Microbiol Biotechnol 56:17–34

Zhang M, Cui SW, Cheung PCK, Wang Q (2007) Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. Trends Food Sci Technol 18(1):4–19

Zhang C, Xiao G, Peng L, Su H, Tan T (2013) The anaerobic co-digestion of food waste and cattle manure. Bioresour Technol 5(129C):170–176

Zheng L, Zheng P, Sun Z, Bai Y, Wang J, Guo X (2007) Production of vanillin from waste residue of rice bran oil by *Aspergillus niger* and *Pycnoporus cinnabarinus*. Bioresour Technol 8(5):1115–1119

Zhong C, Lau MW, Balan V, Dale BE, Yuan YJ (2009) Optimization of enzymatic hydrolysis and ethanol fermentation from AFEX-treated rice straw. Appl Microbiol Biotechnol 84(4):667–676

Zhu H, Suzuki T, Tsygankov AA, Asada Y, Miyake J (1999) Hydrogen production from tofu wastewater by *Rhodobacter sphaeroides* immobilized in agar gels. Int J Hydrog Energy 24:305–310

Ziemiński K, Romanowska I, Kowalska M (2012) Enzymatic pretreatment of lignocellulosic wastes to improve biogas production. Waste Manage 32:1131–1137

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The second generation ethanol production

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1. Abstract

The shortage of fossil energy sources, the alarm over greenhouse gas emissions (GHGs) and global warming have caused an increasing interest in alternative renewable sources of energy, boosting the worldwide trend to produce and use biofuels in substitution of petroleum–based fuels. Bioethanol represents one of the most promising biofuels. The potential yield of first generation bioethanol, produced from traditional agricultural crops, is not sufficient in many parts of the world. Moreover, first generation ethanol production systems pose a concern about competition with food and feed supplies. To overcome these bottlenecks, second generation bioethanol production from non edible renewable lignocellulosic biomass is attracting keen interest. The present manuscript describes the main steps of the second generation bioethanol production process.

2. Introduction

The shortage of energy fossil sources, the alarm over greenhouse gas emissions (GHGs) and global warming [1] have prompted an increasing worldwide interest in alternative renewable sources of energy. The European Parliament with the DIRECTIVE 2009/28/CE — also known as "Climate–Energy package 20–20–20" — has promoted the

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production and use of biofuels in substitution of petroleum–based fuels, establishing a 10% reduction of conventional fuels as the target for 2020.

Bioethanol represents one of the most promising biofuels, exhibiting several advantages, such as high octane number, low cetane number and high heat of vaporization [2]. A variety of biomass feedstock have been explored for ethanol production and can be classified into three types:

a) lignocellulosic materials such as woody biomass, herbaceous perennials and various wastes;
b) starch–rich crops such as maize and grain sorghum;
c) sucrose–rich crops such as sugarcane and sugar beet [3].

First generation bioethanol is produced from traditional agricultural crops, mainly represented by corn, sugarcane and sugar beet. Brazil and USA are the leaders of ethanol production, together accounting for about 90% of the world production. During 2012, the USA has produced 13.3 billion gallons of ethanol [4]. More than 95% of the ethanol produced in the USA for transportation is currently obtained from corn starch and represents 10% of the fuel supply and 25% of all the motor fuel produced [5]. Nowadays, Brazil is the only country that uses ethanol, produced exclusively from sugarcane, as a full substitute for gasoline [6].

The current production of first generation ethanol in the European Union is less than its consumption. Given that the potential yield is not sufficient to accomplish the prefixed European goals, an increase in ethanol importations can be foreseen. Moreover, first generation ethanol production systems pose a concern about the competition with food and feed supplies.

To overcome these bottlenecks, second generation bioethanol production from non edible renewable lignocellulosic biomass is attracting keen interest. Lignocellulosic biomasses are the most abundant renewable resources on Earth. Thus, their utilization for second generation ethanol production would minimize the conflict between land use for food (and feed) and energy production. Moreover, these raw materials are less expensive and present a more even geographical distribution than the conventional agricultural feedstock. A large

fraction of lignocelluloses is represented by residual biomass such as agro–industrial wastes, agricultural and forest crop residues and the organic and paper fractions of municipal solid waste (MSW) that would represent the key response to the need of increasing renewable energy production particularly in the Mediterranean basin [7]. It is worth noting that only small amounts of cellulose, hemicellulose and lignin composing agricultural residues are currently exploited, as the majority is considered waste. Moreover, second generation ethanol production and use has lower green–house gas emissions (<86%) than the first generation fuels, reducing environmental impact, particularly on climate change.

Lignocellulose consists of three types of polymers — cellulose, hemicellulose and lignin — bonded by both non–covalent and covalent cross linkages [8, 9]. Cellulose is a highly crystalline linear polymer composed of D–glucose units linked by β–1,4 glycosidic bonds [8]. Hemicellulose is likewise a polysaccharide, accounting for 25–35% of dry wood [3]. It is a very heterogeneous and ramified polymer, consisting of a mixture of various monosaccharides, such as xylose and arabinose (both 5–carbon sugars) and glucose, mannose and galactose (all 6–carbon sugars), and glucoronic acid. Lignin is present in the cellular wall to give structural support, mechanical resistance, impermeability and defence against microbial attack and oxidative stress. It is an amorphous heteropolymer formed by phenylpropane units joined together by non hydrolysable linkages [9].

Bioethanol production from lignocellulosic materials takes place in three phases: the first step consists in breaking the lignin barrier (pretreatment); further step involves the hydrolysis of cellulose and hemicellulose to generate fermentable sugars (saccharification) followed by the fermentation of mixed hexose and pentose sugars to produce ethanol. In this manuscript, the main routes to perform these steps and their components are described.

3. Pretreatment of lignocellulosic materials for ethanol production

The main goals and characteristics of the pretreatment of lignocellulosic materials are to:

a) remove the barrier of lignin,
b) expose plant cell wall polysaccharides,
c) prevent a great degradation or loss of carbohydrate,
d) minimize the concentration of by–products that are inhibitory to the following hydrolysis and fermentation processes, and (4) be economic and environmentally friendly. Numerous strategies of pretreatment have been developed belonging to different categories, i.e. physical (Pyrolysis, Microwave), physicochemical (Steam Explosion, Ammonia Fiber Explosion), chemical (Alkaline Hydrolysis, Acid Hydrolysis, Ozonolysis, Organosolv) and biological.

3.1. *Physical processes*

Pyrolysis

The pyrolysis pretreatment involves the decomposition of cellulose into H_2, CO, and residual char by using temperatures higher than 300 °C; the process is enhanced when carried out in the presence of oxygen. The wide variety of pyrolysis products points to the need of separating and purifying them prior to use [10]. The recovered solution, after separation from the residual char is mainly composed of cellulose, which can be saccharified for the fermentation of ethanol. Different kind of food crops like corn, sugarcane and soybean were pretreated through pyrolysis to generate ethanol [11]. Ethanol can be produced on a large scale using bio–oil hydrolysate produced by fast pyrolysis of loblolly pine particles. The mainly issue is the presence of inhibitor compounds. These can be removed by economically friendly methods (activated carbon, air stripping and microbial), followed by microbial fermentation, reaching 0.4 g ethanol/g glucose produced with a 79% yield [12]. Pyrolytic sugars from poplar were separated from phenols by solvent extraction and were hydrolyzed into glucose using H_2SO_4 as catalyst. The fermentation of poplar hydrolyzed to produce ethanol was carried out by *Saccharomyces cerevisiae*. The yield obtained was 0.473 g ethanol/g glucose [13].

Microwave

The waves, with frequencies between 0.3 GHz and 300 GHz, are absorbed by water, fats and sugars and their energy is transferred to organic molecules generating heat. This leads to the degradation of lignin, making cellulose and hemicellulose more accessible to enzymatic hydrolysis [14]. Lignocellulose–rich sweet sorghum biogases were pretreated and hydrolysed at the same time using microwave irradiation. An ethanol yield based on total sugar of 480 g kg^{-1} was obtained after 24 h of fermentation using a mixed culture of microorganisms [15]. Microwave pretreatment of oil palm empty fruit bunch beer, combined with alkaline conditions, was reported by Nomanbhay *et al.* [16]. A loss of 74% lignin, 24.5% holocellulose and a yield of total reducing sugars of 41% was obtained at the best operative conditions of 3% (w/v) NaOH at 180 W for 12 min [16].

3.2. *Physicochemical processes*

Steam Explosion

The steam explosion is one of the most currently used methods for the pretreatment of lignocellulosic biomasses. The high temperature (180 to 240 °C) combined to pressure from 1 to 3.5 MPa, followed by an explosive decompression allows one to obtain the breakdown of the rigid lignin structure fibers. The result is a better accessibility of cellulose for enzymatic hydrolysis and fermentation [17]. The optimal steam explosion conditions for sugarcane bagasse are 215 °C for 5 min at maximum operating pressure [18]. After pretreatment and enzymatic saccharification, an overall glucose yield of 86.8% of the content in raw material was achieved. The steam explosion of canola straw increases the glucose recovery in the saccharification process, reaching an increment of 153.22% compared to the control samples, as shown by Garmakhany *et al.* [19].

Ammonia Fiber Explosion (AFEX)

The process consists of treatment with liquid anhydrous ammonia at high temperature (60–100 °C) and pressure (250–300 psi) for 5 min, fol-

lowed by a rapid decrease of the pressure. This causes the disruption of the lignocellulosic structure and the de–crystallization of cellulose, but the lignin remains unchanged; the lignocellulosic biomasses characterized by a higher content of lignin are not efficiently pretreated with this method [20]. The AFEX pretreatment of lignocellulosic materials derived from forages and agricultural residues was performed by Belkacemi *et al.* [21]. The saccharification of the pretreated materials, without any detoxification, allows one to obtain a 60 to 80% of theoretical yield of sugars. Subsequent ethanol fermentation of the hydrolysate by *Pachysolen tannophilus* ATCC 32691 resulted in a theoretical yield of 40–60% after 24 h. AFEX followed by enzymatic hydrolysis has been applied to coastal Bermuda grass by Myoung *et al.* [22]. Pretreatment at 100 °C for 30 min produced 94.8% of theoretical sugar yield, corresponding to the maximum sugar yield achieved with the AFEX treatment.

3.3. *Chemical processes*

Alkaline Hydrolysis

Alkaline hydrolysis (mainly lime ($CaO/Ca(OH)_2$), NaOH, Na_2CO_3) is effective in removing lignin and improving the subsequent enzymatic hydrolysis of the pretreated biomass. Its advantage with respect to other pretreatment technologies is the use of lower temperatures and pressures; however, it requires more time, hours or days rather than minutes or seconds. Compared with the other kinds of pretreatment, alkaline hydrolysis represents a slow process, requires neutralization, and the recovery of added alkali [23]. The optimization of alkaline pretreatment of coffee pulp for ethanol fermentation was performed by Menezes *et al.* [24]. The pretreatment using 4% (w/v) NaOH for 25 min gave the best results with a recovery of cellulose pulp of 69.18% and the production of 13.66 g/L of ethanol with a yield of 0.4 g ethanol/g glucose after the fermentation step. Alvarez *et al.* [25] used alkaline hydrolysis to pretreat different kinds of pine residues. They showed that the hydrolysis yield mainly depends on temperature and alkali concentration. Among the tested operative conditions, 2.5% NaOH for 90 min at 120 °C resulted in the best yield of glucose (41.33% w/w) after enzymatic hydrolysis.

The process could theoretically produce a maximum yield of 90.19% of ethanol/substrate (glucose) and about 80 L of bioethanol per dry ton of woody biomass.

Acid Hydrolysis

Acid hydrolysis represents a widely used pretreatment method because the use of concentrated or diluted acids (usually between 0.2% and 2.5% w/w) at temperatures between 130–210 °C allows one to obtain high yields of sugars [26]. The hemicellulose, easier to be hydrolyzed than cellulose, is attacked by acid solutions [20]. Depending on the reaction conditions, such as temperature, acid concentration and hydrolysis time, the amount of sugar decomposition products, e.g. furfural and 5–hydromethylfurfural (5–HMF), that inhibit the subsequent ethanol fermentation, changes [27]. The hydrolysate obtained by the sulphuric acid pretreatment of *Curcuma longa* waste was directly fermented without a detoxification step as shown by Nguyen *et al.* [28]. The acid hydrolysis was carried out at 122.68 °C with 4.91% sulphuric acid for 50 min, obtaining an ethanol yield of 30.57%. Some acid compounds can be used as pretreatment catalysts, but also as a nitrogen source in the fermentation process for bioethanol production. Rice straw was pretreated with 0.65% nitric acid at 158.8 °C for 5.86 min as reported by Kim *et al.* [29]. After neutralization, the pretreated rice straw was used in the fermentation run without adding any nitrogen sources, reaching an ethanol yield from 10.92 g/L to 14.50 g/L by using *Pichia stipitis*.

Ozonolysis

Ozone attacks the aromatic rings of the lignin structure without damaging hemicellulose and cellulose. The process is affected by ozone concentration, biomass type and moisture content [14]. The ozonolysis of sugarcane bagasse in a fixed bed reactor at room temperature was performed by Travaini *et al.* [30] to evaluate the best samples moisture and ozone concentrations for sugar saccharification. The ozonolysis, under the best experimental conditions (40% sample moisture and 3.44% ozone), increased fermentable carbohydrate release considerably during enzymatic hydrolysis. Glucose and

xylose yields increased from 6.64% and 2.05%, for raw bagasse, to 41.79% and 52.44% for pretreated bagasse. The ozonolysis process is relatively expensive due to large requirements of ozone, but has some advantages: high dry matter concentrations (45–60%), very low production of inhibitory products, and reactions performed at atmospheric conditions [31]. The negative issues are the ineffective removal of lignin and the low yields of reducing sugars. However, combination of ozonolysis with other pretreatment processes has shown promising results. Ozonolysis was studied separately and in combination with wet disk milling (WDM) for the pretreatment of sugarcane bagasse and straw, with the aim of improving their enzymatic saccharification. The use of WDM followed by ozonolysis resulted in glucose yields of 81.1% for bagasse and 92.4% for straw, with shorter WDM times [32].

Organosolv

The use of organic solvent or mixtures of solvents in combination with water causes hydrolysis of the internal bonds in lignin and also of those between lignin and hemicellulose. Ethanol, methanol, acetone, and ethylene glycol are the solvents commonly used in the process. Besides the use of solvents, high temperatures (200 °C) are required, but depending on the type of biomass and the catalyst used, lower temperatures can be sufficient. It could be possible to use organic and inorganic acids as catalysts [14]. Geng *et al.* [33] showed that the addition of an acid catalyst in the organosolv pretreatment of horticultural waste, followed by H_2O_2 post pretreatment, was feasible. The enzymatic hydrolysis of the pretreated waste resulted in a hydrolysate containing 26.9 g/L reducing sugar, while the following fermentation by using *Saccharomyces cerevisiae* produced 11.69 g/L ethanol. Different organosolv operative conditions for the pretreatment of rice straw were tested by Amiri *et al.* [34], the best conditions resulting in 75% (v/v) aqueous ethanol containing 1% w/w sulphuric acid at 180 °C for 30 min. The enzymatic hydrolysis of the pretreated straw allowed one to obtain a glucose yield of 46.2%, which was then fermented to 22.5 g ethanol by *Clostridium acetobutylicum* NRRL B–591.

3.4. *Biological processes*

Biological pretreatment can represent the most environmentally friendly method for the delignification of lignocellulosic biomasses, since it does not require energy and chemical compounds. Although biological pretreatment is considered a promising technology because of its environmental advantages, the process has not been largely applied because it requires a long time (about 3–5 months), results in very high feedstock loss during pretreatment and because the rate of following hydrolysis is very low [35]. The best effective microorganisms that can be employed in the biological delignification are the brown and white rot fungi, which synthesize ligninolytic enzymes. Brown rot attacks cellulose while white and soft rots attack both cellulose and lignin [14]. The biological pretreatment of the rubber wood (*Hevea brasiliensis*) with the white rot fungus *Ceriporiopsis subvermispora* was investigated by Nazarpour *et al.* [36]. After 90 days of fermentation, the lignin and hemicelluloses loss was 45.06% and 42.08%, respectively, while the loss of cellulose was very low (9.50%). This treatment increased the sugar yield to about 27.67% during the subsequently hydrolysis. The hydrolysate obtained from wheat straw pretreated with the white–rot fungus *Irpex lacteus* was fermented by the yeast *Pachysolen tannophilus*. The ethanol yield obtained was between 23 and 35% greater than the yields typically obtained with a conventional process, in which wheat straw is pretreated using steam explosion and fermented with the yeast *Saccharomyces cerevisiae* [37].

4. Hydrolysis of polysaccharides fraction of lignocelluloses for ethanol production

After pretreatment, the released cellulose and hemicelluloses are hydrolyzed into fermentable monomeric sugars (hexoses and pentoses). The hydrolysis of (hemi)cellulose remains a major bottleneck for the efficient production of ethanol [38, 39].

Two major methods can be employed to carry out the hydrolysis process. The first method involves acids as catalysts, while the second uses enzymes known as cellulases [40, 41]. The acid hydrolysis degrades the cellulose and hemicelluloses polymers into monomeric sugars with-

out preliminary pretreatment of biomass. It can be carried out using concentrated or diluted acids. The concentrated acid hydrolysis, with sulphuric and hydrochloric acids, is the mainly used method for the hydrolysis of lignocellulosic biomass [27] producing high hydrolysis yields of cellulose [40]. However, this process requires large quantities of acids, expensive acid recycling and causes degradation of monosaccharides.

It follows that enzymatic hydrolysis is the generally preferred method due to the mild operation conditions, higher conversion yields, low energy cost, and less corrosive and toxic conditions compared to the acid hydrolysis [20, 42, and 43]. However, the enzymes involved in the enzymatic hydrolysis have higher costs and this represent the main bottleneck in lignocellulosic ethanol production [44].

4.1. *Enzymatic hydrolysis*

As reviewed in Kumar and Murthy [42], the enzymatic hydrolysis process can be divided into two phases. The first step is represented by enzymatic depolymerization in which long polysaccharide chains are hydrolyzed to soluble oligomers. In the second step, oligomers are hydrolyzed to sugar monomers. The rate–limiting step in the process is considered the first hydrolysis phase.

Due to the complexity of the lignocellulosic carbohydrates, the activities of multiple hydrolytic enzymes are required for complete deconstruction of the various components of the lignocellulosic biomass; these enzymes are produced as either *free cellulases* or *complexed cellulases*, called cellulosomes [43, 45]. The efficient enzyme mixture comprises ten different enzymes. Among these, at least 6 can be considered crucial, depending on the composition of the raw materials [46]. Enzymes involved in lignocellulosic biomass are following described.

Cellulase enzymes

Cellulases are glycosyl hydrolases (GH) involved in cellulose hydrolysis, whose classification is available on the CArbohydrate–Active EnZymes web site (CAZY: www.cazy.org). These enzymes are con-

ventionally divided in three major classes: endoglucanases, exoglucanases and β–glucosidases [44, 47, and 48].

Endoglucanases (EG) or 1,4–beta–glucanases (EC 3.2.1.4) randomly hydrolyze intramolecular β–1,4–glucosidic linkages, creating new chain–ends; exoglucanases (CBH) or cellobiohydrolases (EC 3.2.91) hydrolyze the 1,4–glycocidyl linkages from the reducing or non–reducing ends to form cellobiose; β–glucosidases (BG) (EC 3.2.1.21) hydrolyze cello–oligosaccharides and cellobiose into glucose eliminating cellobiose inhibition.

Fungi and bacteria have been reported to be able to produce cellulases. In particular, the fungus *Trichoderma reesei* is the preferred industrial source of cellulases and hemicellulases due to the high level of secreted enzymes. This fungus produces at least two CBHs, five EGs, and two BGs whose expression is finely regulated [49]. However, this fungus produces low levels of BGs if compared to *Aspergillus sp.* *Trichoderma* strains engineered with extra β–glucosidase have been studied. In particular Nakazawa *et al.*[50] reported a recombinant *T. reesei* strain expressing *A. Aculeatus* BGL1. The resulting strain appears more suitable for cellulose hydrolysis. Among bacteria, several *Bacillus* strains isolated from natural habitats were shown to be able to produce cellulase activities [51].

Cellulosome systems are multi–enzymatic complexes produced mainly by anaerobic bacteria but also by other bacteria and few anaerobic fungi [40, 48, and 52]. In these systems there are two subunit types, the scaffolding and the enzymatic subunits. The structural scaffolding subunits contain a carbohydrate–binding module that binds the cellulose surface and multiple copies of cohesins (named dockerins) that interact with the enzymatic subunits, CBD (cellulose binding domains) and CBM (carbohydrates binding modules).

Hemicellulase enzymes

In addition to the three major groups of cellulose enzymes, there is also a number of hemicellulases which hydrolyze hemicellulose. Due to the more varied composition of this polysaccharide, enzymes degrading hemicellulose are divided into two major groups: depolymerising enzymes, which cleave the backbone, and enzymes that remove substituents. These enzymes act synergistically to hydrolyze lignocellulosic polysaccharides [48].

Xylanase

Most studies on hemicellulases were focused on enzymes that hydrolyze xylan. According to the CAZY database, these enzymes include GH 3, 30, 39, 43, 52, 54, 116 and 120.

The complete degradation of xylan requires the cooperative action of endoxylanase and xylosidase.

Endo–1,4–β–xylanase (1,4–b–d–xylan xylanohydrolases, EC 3.2.1.8) cleaves the glycosidic bonds in the xylan backbone releasing shorter xylo–oligosaccharides; β–xylosidase (1,4–b–d–xylan xylohydrolase, EC 3.2.1.37) cleaves the small xylo–oligosaccharides and cellobiose into xylose. These enzymes are produced by several microorganisms such as fungi, bacteria, yeast, and marine algae. Filamentous fungi are interesting producers because the enzymes are secreted at levels higher than those of yeasts and bacteria.

As reviewed by Van Dyk and Pletschke, [48] endoxylanases have different specificities. For example, family 11 xylanases prefer cleaving the xylan backbone in unsubstituted regions, while family 10 xylanases are able to cleave the xylan backbone closer to the substituents.

The synergic activity of several accessory enzymes which remove the various substituents linked to the backbone is also essential.

α–arabinofuranosidase

α–L–arabinofuranosidase (EC 3.2.1.55) removes the L–arabinose substituents from the xylan backbone. According to the CAZY database, arabinofuranosidases are present in GH 3, 43, 51, 54 and 62. All these families, except for GH 43 and 62, perform hydrolysis with retention of the anomeric configuration.

As reviewed by Van Dyk and Pletschke [48], α–L– arabinofuranosidases have different specificities cleaving 1,2 linkages or 1,3 linkages. Moreover, these enzymes are able to cleave doubly substituted arabinose residues from arabinoxylan. As reported by Lagaert et al [53], complete degradation of arabinoxylan needs the synergistic action of arabinofuranosidases and also of β–xylosidases. Rasmussen *et al.* [54] demonstrated that optimal hydrolysis of soluble wheat arabinoxylan requires the combined action of an endoxylanase, a β–xylosidase and the two types of arabinofuranosidases.

α–glucuronidase

α–glucuronidase (EC 3.2.1.139) removes 4–O–methyl glucuronic acid substituents from the glucuronoxylan. This enzyme hydrolyses the α–1,2 bonds between the glucuronic acid residues and β–D–xylopyranosyl backbone units [44].

Esterases

Esterases hydrolyse the ester linkages between xylose units of the xylan and acetic acid (Acetylxylan esterase, EC 3.1.1.72) or between arabinose side chain residues and phenolic acids such as p–coumaric acid (p–coumaric acid esterase EC 3.2.1.73) or ferulic acid (ferulic acid esterase, EC 3.2.1.73).

Acetylxylan esterase removes the O–acetyl groups from positions 2 and/or 3 on the β–D–xylopyranosyl residues of acetyl xylan.

Ferulic acid and p–coumaric acid esterases (EC 3.1.1.73) hydrolyze ester linkages on xylan, liberating the respective phenolic acids linked to the arabinofuranoside residues [48].

Mannanases

Mannose residues have α–1,6–galactose as side groups and these are acetylated at the O–2 and O–3 positions. The core enzymes for complete degradation of mannan into simple sugars are the endomannanase and β–mannosidase.

Endo–β–1,4–mannanases (EC 3.2.1.78) catalyze hydrolysis of the β–1,4–linked backbone within different mannans. In the CAZy database enzymes are classified into three different glycoside hydrolase families: GH5, GH26 and GH113 [55].

β–mannosidase (EC 3.2.1.25) is the key enzyme responsible for catalyzing random hydrolysis of manno–glycosidic bonds in the main chain. All plant mannanases belong to family GH5 subfamily 7 (GH5_7) [56].

Additional enzymes, such as α–galactosidases (EC 3.2.1.22), β– glucosidase (EC 3.2.1.21) and acetyl mannan esterases are required to remove side chain sugars on mannans.

5. Sugars fermentation for bioethanol production

Once lignin has been removed and the saccharification of the free accessible (hemi)cellulose portions of the biomass has been carried out, the final step to be performed is the fermentation of sugars into bioethanol.

Saccharomyces cerevisiae is the most widely used organism for ethanol production from hexoses, whilst *Pichia stipitis* and *Candida shehatae* are the main yeasts able to ferment both hexose and pentose sugars to ethanol. Bacteria belonging to the species *Clostridia* and *Zymomonas*, and fungi such as *Fusarium* spp. have been also demonstrated to be useful for ethanol production.

The current process is optimized for 6–carbon atoms sugars fermentation since most yeasts cannot ferment 5–carbon atoms sugars. Given that the economically competitive ethanol production from lignocellulosic materials requires the efficient use of both hexose and pentose monosaccharides, research is now strongly exploring new engineered yeasts able to ferment 5–carbon sugars with high yields. The main routes for ethanol fermentation are described below.

5.1. *Ethanol production by separate hydrolysis and fermentation (SHF)*

Two main routes can be followed for ethanol production, such as separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).

In SHF, the bioconversion of lignocellulose takes place in two separate reactors, thus separating the saccharification and the fermentation processes, resulting in the quick and easy possibility to optimize each production step, with conduction of each process at the optimal conditions of pH and temperature.

An example of SHF process application for ethanol production is described by Erdei *et al.* [57], who reported an experiment of separate hydrolysis and co–fermentation (combining xylose and glucose fermentation) of steam–pretreated wheat straw (SPWS) combined with wheat starch hydrolysate feed, achieving an average yield of ethanol up to 86%.

5.2. *Ethanol production by simultaneous saccharification and fermentation, SSF*

In SSF, enzymatic hydrolysis and fermentation are carried out together, using a unique reactor. By taking place in the same reactor, this kind of process has a number of advantages such as the reduction of costs and the increase of hydrolysis rate, besides the possibility to make the fermentable sugars immediately available to yeasts.

However, the ideal pH or temperature conditions for the saccharification step may differ from those of the fermentation step. Thus, it is very difficult to find favorable conditions for both processes.

As generally stated, SSF is a much more competitive process in comparison to SHF from the economical point of view, since the use of a unique bioreactor results in a total reduction of investment and operational costs.

Recent works regarding SSF are those by Nahar and Pryor [58] and Rezic´ *et al.* [59], who used as feedstock for bioethanol production crushed whole sugar beets (ethanol yield up to 92%) and sugar beet pulp (ethanol yield up to 49%), respectively.

Hydrolysis and fermentation conditions for production of ethanol from very high–gravity cassava mash during a simultaneous saccharification and fermentation (SSF) process were optimized using a statistical methodology [60], reaching a final ethanol yield of 15.03% (wt.%) in 72 h.

5.3. *Ethanol production by consolidated bio processing, CBP*

Consolidated bio processing (CBP) has been so far recognized as the best system to reduce the cost of biomass processing, by combining the hydrolysis of the polysaccharides and the subsequent fermentation of the hexoses/pentose sugars.

Differently from SSF, besides the use of a unique reactor, in CBP a single engineered microorganism is used, which is able to directly convert (hemi)cellulose into ethanol using its own enzymatic machinery.

So far, there are no microorganisms able to perform both the enzymatic hydrolysis and the fermentative steps with high yield of both processes, thus genetic engineering must be applied to create suitable microorganisms for the CBP process.

It is worth noting that there are two main routes to perform CBP: CBP I route, characterized by the engineering of a cellulase producing microorganism to make it able to ferment sugars; CBP II route aimed at the engineering of an ethanologenic microorganism to make it able to produce cellulases or hemicellulases.

As recently reviewed by Olson *et al.* [39], progress in the development of genetic tools for fungal systems has been obtained, focusing mostly on the increase of cellulase production, especially for fungi like *Fusarium oxysporum* and *Trichoderma reesei*. There has also been a substantial progress in the development of genetic tools for free–enzyme bacterial systems, including *Clostridium phytofermentans*, *Clostridium japonicas*, *Thermoanaerobacter* and *Thermanaero–bacterium* sp. The latter, a thermophilic anaerobe that utilizes a broad range of substrates including xylan, is a prominent example of engineered organisms with recently developed genetic tools to produce a biofuel at high yield [61, 62].

CBP category I

Trichoderma, Aspergillus, *Rhizopus* and *Fusarium* are the potential filamentous fungi suitable for development of CBP type I. All of them produce high amounts of both cellulases and hemicellulases, leading to high yields of fermentable sugars. Particularly, *T. reesei* is the best producer of such activities, so far described.

These fungi have been reported as ethanol producers, even if with very low yield and high concentration of by–products, such as lactic acid.

As reviewed by Amore and Faraco [63], many efforts are focused on the elucidation of regulatory mechanisms, in order to advance the knowledge on the metabolic pathways involved in ethanol production by filamentous fungi recognized as potential candidates for CBP type I.

CBP category II

S. cerevisiae is the most used yeast for ethanol production from C6 sugars and it represents the best candidate for CBP type II. Thus, a big challenge is to confer to this microorganism the capability to produce cellulase and/or hemicellulase activities.

S. cerevisiae has been so far described as a suitable host for recombinant production of different enzymes, among which are also enzymes involved in the conversion of polysaccharides.

As previously reported, for the complete conversion of cellulose there is the need of three different enzymes namely endoglucanases, cellobiohydrolases and β–glucosidases, whose most common sources are fungi, especially *Trichoderma reesei*, and bacteria.

Cellobiohydrolases (CBH) and endoglucanases have been successfully expressed in *S. cerevisiae*, whilst very low yields of fungal β–glucosidase have been achieved.

Kluyveromyces marxianus is another interesting candidate for CBP II. It has been used with good results as host for heterologous proteins, including enzymes involved in cellulose hydrolysis.

Among bacteria, *E. coli* represents the main candidate for the CBP II category. It is able to metabolize a wide spectrum of sugars and a well–know microorganism to be genetically engineered.

Despite the extensive research performed to optimize *E. coli* recombinant cellulase production and reduce the addition of external cellulases, the potential of the obtained recombinant strains to directly grow on plant biomass cannot be proved, mainly due to the complex cocktail of enzyme needed for the complete lignocelluloses conversion into fermentable sugar, as reviewed by Amore *et al.* [64].

Zymomonas mobilis, *Klebsiella oxytoca* and *Bacillus subtilis* are other candidates for the CBP II process, being known for ethanol production and tolerance to the toxicity of the final products.

6. Conclusions

To overcome bottlenecks associated to first generation bioethanol, produced from traditional agricultural crops, second generation bioethanol production from non edible renewable lignocellulosic biomass is attracting strong interest, as it minimizes the conflict between land use for food (and feed) and energy production. Lignocelluloses are the most abundant renewable resources on Earth and include residual biomass such as agro–industrial wastes, agricultural and forest crop residues and the organic and paper fractions of municipal solid waste (MSW). Moreover, second generation ethanol

production and use show lower green–house gas emissions than the first generation fuels, reducing environmental impact. However, the process currently adopted for second generation bioethanol production is not competitive as yet. Lignocellulose consists of three types of polymers — cellulose, hemicellulose and lignin — bonded by both non–covalent and covalent cross linkages. Bioethanol production from lignocellulosic materials takes place in three phases, namely a pretreatment step to remove the lignin barrier, hydrolysis of cellulose and hemicellulose to generate fermentable sugars (saccharification) and fermentation of mixed hexose and pentose sugars to produce ethanol.

As regards the pretreatment step, all the techniques so far used (physical, physico–chemical and chemical processes), are not eco–friendly and generate inhibitors which negatively affect the next step of lignocellulose saccharification into fermentable sugars. Biological pretreatment could represent a valid alternative, but the long treatment time and the low final yield do not make it a feasible process, as yet.

Despite the high costs of enzyme production, enzymatic hydrolysis is preferred to the acid one. A wide spectrum of enzymes is required to completely convert pretreated lignocellulose into fermentable sugars, both cellulases, acting on the cellulose polymer for C6 sugars release, and hemicellulases, for C5 sugars production.

Simultaneous saccharification and fermentation (SSF) and separate hydrolysis and fermentation (SHF) are the main routes used to produce bioethanol by fermenting the hydrolysate from cellulose, even if consolidated bioprocessing (CBP) has been recognized as the best system to reduce the cost of biomass processing, by combining the hydrolysis of the polisaccharides and the subsequent fermentation of the hexose/pentose sugars.

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References

[1] MacCracken M.C., 2008. *Prospects for future climate change and the reasons for early action*. Journal of the Air & Waste Management Association, 58(6), 735–86.

[2] Balat M., 2007. *Global bio–fuel processing and production trends*. Energy, Exploration & Exploitation, 25(3), 195–218.

[3] Balat M., H. Balat, O.Z. Cahide, 2008. *Progress in bioethanol processing*. Progress in Energy and Combustion Science, 34, 551–573.

[4] Renewable Fuels Association (RFA). *2012 Ethanol industry Outlook*.

[5] Renewable Fuels Association (RFA). *2013 Ethanol industry Outlook*.

[6] Soccol C.R., L.P. Vandenberghe, A.B. Medeiros, S.G. Karp, M. Buckeridge, L.P. Ramos, A.P. Pitarelo, V. Ferreira–Leit ao, L.M. Gottschalk, M.A. Ferrara, E.P. da Silva Bon, L.M. de Moraes, A. Araújo Jde, F.A. Torres, 2010. *Bioethanol from lignocelluloses: Status and perspectives in Brazil*. Bioresource Technology, 101(13), 4820–4825.

[7] Faraco V., Y. Hadar, 2011. *The potential of lignocellulosic ethanol production in the Mediterranean Basin*. Renewable & Sustainable Energy, 15(1), 252–266.

[8] Pérez J., J. Mu noz–Dorado, T. De–la–Rubia, J. Martínez, 2002. *Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview*. International Microbiology, 5, 53–63.

[9] Sanchez C., 2009. *Lignocellulosic residues: Biodegradation and bioconversion by fungi*. Biotechnology Advances, 27, 185–194.

[10] Shiung Lam S., H.A. Chase, 2012. *A review on waste to energy processes using microwave pyrolysis*. Energies, 5, 4209–4232.

[11] Rogers, J.G., Brammer J.G., 2012. *Estimation of the production cost of fast pyrolysis bio– oil*. Biomass Bioenergy, 36, 208–217.

[12] Wang H., D. Livingston , R. Srinivasan , Q. Li, P. Steele, F. Yu, 2012. *Detoxification and fermentation of pyrolytic sugar for ethanol production*. Ap-

plied Biochemistry and Biotechnology, 168, 1568–1583.

[13] Lian J., S. Chen, S. Zhou, Z. Wang, J. O'Fallon, C.Z. Li, M. Garcia–Perez, 2010. *Separation, hydrolysis and fermentation of pyrolytic sugars to produce ethanol and lipids*. Bioresource Technology, 101(24):9688–9699.

[14] Chaturvedi V., P. Verma, 2013. *An overview of key pretreatment processes employed for bioconversion of lignocellulosic biomass into biofuels and value added products*. Biotech, 3, 415–431.

[15] Marx S., B. Ndaba, I. Chiyanzu, C. Schabort, 2013. *Fuel ethanol production from sweet sorghum bagasse using microwave irradiation*. Biomass and Bioenergy, xxx, 1–6.

[16] Nomanbhay S.M., R. Hussain, K. Palanisamy, 2013. *Microwave assisted enzymatic saccharification of oil palm empty fruit bunch fiber for enhanced fermentable sugar yield*. Journal of Sustainable Bioenergy Systems, 3, 7–17.

[17] Stelte W., 2013. *Steam explosion for biomass pretreatment*. Danish Technological Insistute, Taastrup, DK, 15 pp.

[18] Amores I., I. Ballesteros, P. Manzanares, F. Sáez, G. Michelena, 2013. *Ethanol production from sugarcane bagasse pretreated by steam explosion*. Electronic Journal of Energy & Environment, 1, 25–36.

[19] Garmakhany A. D., M. Kashaninejad, M. Aalami, Y. Maghsoudlou, M. Khomieri, L. G. Tabil., 2013. *Enhanced biomass delignification and enzymatic saccharification of canola straw by steam–explosion pretreatment*. Journal of the Science of Food and Agriculture, DOI 10.1002/jsfa.6466.

[20] Sarkar N., S.K. Ghosh, S. Bannerjee, K. Aikat, 2012. *Bioethanol production from agricultural wastes: An overview*. Renewable Energy, 37, 9–27.

[21] Belkacemi K., G. Turcoitte, D. De Halleux, P. Savoie, 1998. *Ethanol production from AFEX– treated forages and agricultural residues*. Applied Biochemistry and Biotechnology, 70(72), 441–462.

[22] Myoung Lee J., H. Jameel, R.A. Venditti, 2010. *A comparison of the autohydrolysis and ammonia fiber explosion (AFEX) pretreatments on the subsequent enzymatic hydrolysis of coastal Bermuda grass*. Bioresource Technology, 101, 5449–5458.

[23] Chang V.S., M. Nagwani, C.H. Kim, M.T. Holtzapple, 2001. *Oxidative lime pretreatment of high–lignin biomass poplar wood and newspaper*. Applied Biochemistry and Biotechnology, 94, 1–28.

[24] Menezes E.G.T., J.R. do Carmo, J.G.L.F. Alves, A.G.T. Menezes, I.C. Guimar aes, F. Queiroz, C.J. Pimenta, 2014. *Optimization of alkaline pre-*

treatment of coffee pulp for production of bioethanol. Biotechnology Progress, 0, 1–12.

[25] Alvarez C.E.S., J.L. Miranda, M.R. Castro, G.P. Verdín, M.A.R. Pérez, I.C. Hernández, 2013. *Alkaline pretreatment of Mexican pine residues for bioethanol production*. African Jurnal of Biotechnology, 12, 4956–4965.

[26] Grohmann K., R. Torget, M. Himmel, 1986. *Dilute acid pretreatment of biomass at high solids concentrations*. Biotechnology and Bioengineering symposium, 17:137–151.

[27] Lenihan P., A. Orozco, E. O'Neill, M.N.M. Ahmad, D.W. Rooney, G.M. Walker, 2010. *Dilute acid hydrolysis of lignocellulosic biomass*. Chemical Engineering Journal, 156, 395–403.

[28] Nguyen C.M., T.N. Nguyen, G.J. Choi, Y.H. Choi, K.S. Jang, Y.J. Park, J.C. Kim, 2014. *Acid hydrolysis of Curcuma longa residue for ethanol and lactic acid fermentation*. Bioresource Technology, 151, 227–235.

[29] Kim I., B. Lee, J.Y. Park, S.A. Choi, J.I. Han, 2014. *Effect of nitric acid on pretreatment and fermentation for enhancing ethanol production of rice straw*. Carbohydrate Polymers, 99, 563– 567.

[30] Travaini R., M.D.M. Otero, M. Coca, R. Da–Silva, S. Bolado, 2013. *Sugarcane bagasse ozonolysis pretreatment: effect on enzymatic digestibility and inhibitory compound formation*. Bioresource Technology, 133, 332–339.

[31] Bensah E.C., M. Mensah, 2013. *Chemical pretreatment methods for the production of cellulosic ethanol: technologies and innovations*. International Journal of Chemical Engineering. doi.org/10.1155/2013/719607.

[32] de Barros R.R.O., R.S. Paredes, T. Endo, E.P. da Silva Bon, S.H. Lee, 2013. *Association of wet disk milling and ozonolysis as pretreatment for enzymatic saccharification of sugarcane bagasse and straw*. Bioresource Technology, 136, 288–294.

[33] Geng A., F. Xin, J.Y. Ip, 2013. *Ethanol production from horticultural waste treated by a modified organosolv method*. Bioresource Technology, 104, 715–721.

[34] Amiri H., K. Karimi, H. Zilouei, 2014. *Organosolv pretreatment of rice straw for efficient acetone, butanol, and ethanol production*. Bioresource Technology, 152, 450–456.

[35] Sun Y., J. Cheng, 2002. *Hydrolysis of lignocellulosic material for ethanol production: a review*. Bioresource Technology, 83, 1–11.

[36] Nazarpour F., D.K. Abdullah, N. Abdullah, R. Zamiri, 2013. *Evaluation of biological pretreatment of rubberwood with white rot fungi for enzy-*

matic hydrolysis. Materials, 6, 2059– 2073.

[37] López–Abelairas M., T.A. Lu–Chau, J.M. Lema, 2013. *Fermentation of biologically pretreated wheat straw for ethanol production: comparison of fermentative microorganisms and process configurations*. Applied Biochemistry and Biotechnology, 170, 1838–1852.

[38] MatanoY., T. Hasunuma, A. Kondo, 2012. *Display of cellulases on the cell surface of Saccharomyces cerevisiae for high yield ethanol production from high–solid lignocellulosic biomass*. Bioresource Technology , 108, 128–133.

[39] Olson D.G, J.E. McBride, A.W. Shaw, L.R. Lynd, 2011. *Recent progress in consolidated bioprocessing*. Current Opinion in Biotechnology 23, 1–10.

[40] Verardi A., I. De Bari, E. Ricca, V. Calabrò, 2012. *Hydrolysis of Lignocellulosic Biomass: Current Status of Processes and Technologies and Future Perspectives*. In: Bioethanol (M.A. Pinheiro Lima ed.), InTech, 95–122.

[41] Limayem A., S.C. Ricke, 2012. *Lignocellulosic biomass for bioethanol production: Current perspectives, potential issues and future prospects*. Progress in Energy and Combustion Science, 38, 449–467.

[42] Kumar D., G.S. Murthy, 2013. *Stochastic molecular model of enzymatic hydrolysis of cellulose for ethanol production*. Biotechnology for Biofuel, 6:63.

[43] Yang B., Z. Dai, S.Y. Ding, C.E. Wyman, 2011. *Enzymatic hydrolysis of cellulosic biomass*. Biofuels, 2(4), 421–450.

[44] Binod P., K.U. Janu, R. Sindhu, A. Pandey, 2011. *Hydrolysis of Lignocellulosic Biomass for Bioethanol Production*. In: Biofuels (A. Pandey, C. Larroche, S.C. Ricke, C.G. Dussap, E. Gnansounou eds.) Burlington: Academic Press, 229–250.

[45] Chandel A.K, 4, G. Chandrasekhar, K. Radhika, R. Ravinder, P. Ravindra, 2011. *Bioconversion of pentose sugars into ethanol: A review and future directions*. Biotechnology and Molecular Biology Review, 6(1), 8–20.

[46] Viikari L, J. Vehmaanpera, A. Koivula, 2012. *Lignocellulosic ethanol: From science to industry*. Biomass and bioenergy, 46, 13–24.

[47] Hasunuma T., F.Okazaki, N. Okai, K.Y. Hara, J. Ishii, A. Kondo, 2013. *A review of enzymes and microbes for lignocellulosic biorefinery and the possibility of their application to consolidated bioprocessing technology*. Bioresource Technology, 135, 513–522.

[48] Van Dyk J.S, B.I. Pletschke, 2012. *A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes–Factors affecting enzymes, conversion and synergy*. Biotechnology Advances,

30, 1458–1480.

[49] Amore A, S. Giacobbe, V. Faraco, 2013a. *Regulation of cellulase and hemicellulase gene expression in fungi.* Current Genomics, 14, 1–20.

[50] Nakazawa H., T. Kawai, N. Ida, Y. Shida, Y. Kobayashi, H. Okada, S. Tani, J. Sumitani, T. Kawaguchi, Y. Morikawa, W. Ogasawara, 2011. *Construction of a recombinant Trichoderma reesei strain expressing Aspergillus aculeatus β–glucosidase 1 for efficient biomass conversion.* Biotechnology and Bioengineering, 109, 92–99.

[51] Amore A, O. Pepe, V. Ventorino, A. Aliberti, V. Faraco, 2013b. *Cellulolytic Bacillus strains from natural habitats — A review.* Chimica Oggi — Chemistry Today, 31, 2, 6–9.

[52] Fontes, C.M, H.J. Gilbert, 2010. *Cellulosomes: highly efficient nanomachines designed to deconstruct plant cell wall complex carbohydrates.* Annual Review of Biochemistry, 79, 655–681.

[53] Lagaert S., A. Pollet, C.M. Courtin, G. Volckaert, 2013. *β–Xylosidases and α–L–arabinofuranosidases: Accessory enzymes for arabinoxylan degradation.* Biotechnology Advances, http://dx.doi.org/10.1016/j.biotechadv.2013.11.005.

[54] Rasmussen L.E, C. Xu, J.F. Sørensen, M.K. Nielsen, A.S. Meyer, 2012. *Enzyme kinetics and identification of the rate–limiting step of enzymatic arabinoxylan degradation.* Biochemical Engineering Journal 69, 8–16.

[55] Wang Y., F. Vilaplana, H. Brumer, H. Aspeborg, 2013. *Enzymatic characterization of a glycoside hydrolase family 5 subfamily 7 (GH5_7) mannanase from Arabidopsis thaliana.* Planta, DOI 10.1007/s00425–013–2005–y.

[56] Aspeborg H., P.M. Coutinho, Y. Wang, H. Brumer, B. Henrissat, 2012. *Evolution, substrate specificity and subfamily classification of glycoside hydrolase family 5 (GH5).* BMC Evolutionary Biology, 12, 186.

[57] Erdei B., B. Frankó, M.Galbe, G. Zacchi, 2012. *Separate hydrolysis and co–fermentation for improved xylose utilization in integrated ethanol production from wheat meal and wheat straw.* Biotechnology for Biofuels, 5, 12.

[58] Nahar N., S. Pryor, 2013. *Enzymatic hydrolysis and fermentation of crushed whole sugar beets.* Biomass Bioenergy, 59, 512–519.

[59] Rezič T., D. Oros, I. Marković, D. Kracher, R. Ludwig, B.J. Santek, 2013. *Integrated hydrolyzation and fermentation of sugar beet pulp to bioethanol.* Microbiological Biotechnology, 23 (9), 1244–1252.

[60] Yingling B., Y. Zongcheng, W. Honglin, C. Li, 2010. *Optimization of bioethanol production during simultaneous saccharification and fermentation*

in very highgravity cassava mash. Antonie van Leeuwenhoek, 99, (2), 329–339.

[61] Shaw A.J., D.A. Hogsett, L.R. Lynd, 2010. *Natural competence in Thermoanaerobacter and Thermoanaerobacterium species*. Applied Environmental Microbiology, 76, 4713–4719.

[62] Shaw A.J., S.F. Covalla, D.A. Hogsett, C.D. Herring, 2011. *Marker removal system for Thermoanaerobacterium saccharolyticum and development of a markerless ethanologen*. Applied Environmental Microbiology , 77, 2534–2536.

[63] Amore A., V. Faraco, 2012. *Potential of fungi as category I Consolidated BioProcessing organisms for cellulosic ethanol production*. Renewable and Sustainable Energy Reviews, 16, 3286–3301.

[64] Amore A., S. Giacobbe, V. Faraco, 2013. *Consolidated bioprocessing for improving cellulosic ethanol production*. In: Lignocellulose Conversion, (V. Faraco ed.) Springer–Verlag Berlin Heidelberg, 169–196.

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MINI-REVIEW

Bioreactors for lignocellulose conversion into fermentable sugars for production of high added value products

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Abstract Lignocellulosic biomasses derived from dedicated crops and agro-industrial residual materials are promising renewable resources for the production of fuels and other added value bioproducts. Due to the tolerance to a wide range of environments, the dedicated crops can be cultivated on marginal lands, avoiding conflict with food production and having beneficial effects on the environment. Besides, the agro-industrial residual materials represent an abundant, available, and cheap source of bioproducts that completely cut out the economical and environmental issues related to the cultivation of energy crops. Different processing steps like pretreatment, hydrolysis and microbial fermentation are needed to convert biomass into added value bioproducts. The reactor configuration, the operative conditions, and the operation mode of the conversion processes are crucial parameters for a high yield and productivity of the biomass bioconversion process. This review summarizes the last progresses in the bioreactor field, with main attention on the new configurations and the agitation systems, for conversion of dedicated energy crops (*Arundo donax*) and residual materials (corn stover, wheat straw, mesquite wood, agave bagasse, fruit and citrus peel wastes, sunflower seed hull, switchgrass, poplar sawdust, cogon grass, sugarcane bagasse, sunflower seed hull, and poplar wood) into sugars and ethanol. The main novelty of this review is its focus on reactor components and properties.

Keywords Single and double helical ribbon impeller · Rushton impeller · S-shaped impeller · Anchor impeller · Pitched-blade impeller · Peg-mixer · Paddle blade magnetic impeller

Introduction

The use of crops as renewable sources of energy and compounds in alternative to fossil resources can promote a sustainable development avoiding the problems of shortage of fossil feedstock (Liguori et al. 2013; Kajaste 2014), but it generates problems in the agricultural market since their cultivation increases the lands subtracted to the food production and rising global food prices (Scheidel and Sorman 2012).

To limit the competition between the food- and the non food-crop lands, the cultivation of dedicated energy crops in marginal lands non appropriate for the traditional food crops is spreading throughout the world (Popp et al. 2014). It is noteworthy that the large-scale cultivation of dedicated crops, such as the perennial biomass *Arundo donax*, have favorable effects on the environment, since it improves soil fertility and reduces soil erosion (Fagnano et al. 2015). Moreover, lignocellulosic agro-industrial residual materials represent a further alternative of cheap sources to further minimize the conflict of food versus fuel. They avoid the displacement of food crops and the issues related to the deforestation, limiting the negative impacts on the environment (Iqbal et al. 2013).

Due to the high cellulose and hemicellulose contents (an average of 40 and 30 %, respectively) (Limayem and Ricke 2012), the dedicated crops and the residual materials can be converted in different value-added products, such as

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fermentable sugars (Mezule et al. 2015) and bioethanol or other bioproducts obtained by sugars fermentation (Shahsavarani et al. 2013). The complexity of the lignocellulosic macromolecular structure requires a bioconversion process consisting of three phases (Fig. 1). The first step is the biomass pretreatment needed to remove the lignin and make the polysaccharides more accessible to the further hydrolysis and it is considered generating the most negative impact on the environment, due to the high energetic inputs. The polysaccharides are then subjected to hydrolysis into monosaccharides mainly performed by hydrolytic enzymes during the second step, which is the most costly step of the overall process due to the high costs of the enzymes. In the last fermentation step, the fermentable sugars are converted into the targeted added value bioproducts (Jørgensen et al. 2007a).

Several efforts are under way to improve both the molecular systems, hydrolytic enzymes (Amore et al. 2012, 2013, 2015; Salmon et al. 2014; Weingartner Montibeller et al. 2014; Giacobbe et al. 2014), and microorganisms (Liguori et al. 2015; Ventorino et al. 2015), and bioreactor systems adopted for the biomasses bioconversion process in order to solve the environmental and economical issues of the process (Wang et al. 2011; Khoo 2015).

This review summarizes the last advances in the bioreactor field, with main focus on the new configurations and the agitation systems, for conversion of dedicated energy crops and residual materials into sugars and ethanol by separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and co-fermentation (SSCF) and consolidated bioprocessing (CBP) (Fig. 2).

The attention was focused on the dedicated crops *Arundo donax* (Table 1), corn stover (Table 2), wheat straw (Table 3) and other residual materials (Table 4). The main novelty of this review is its focus on reactor components and properties.

Bioreactors for *Arundo donax* conversion

Production of sugars from SHF

The energy crop *Arundo donax* was investigated by Palmqvist and Lidén (2012), in comparison with spruce, to evaluate the influence of water-insoluble solids (WIS) content on glucose yield during the hydrolysis. Both biomasses were steam pretreated and tested at WIS content of 10, 15, and 20 %. The process was performed in a 3-L stirred tank bioreactor (Belach Bioteknik, Stockholm, Sweden), supplied with an **anchor impeller** (Fig. 3a) (13-cm diameter and 2-cm blade width). The Cellic CTec2 (Novozymes, Denmark) (0.1 g solution g^{-1} WIS) was adopted as enzyme preparation. They tested two different methods, the first keeping constant the impeller speed at 10 rpm, and the second one keeping constant the impeller power input, in order to identify the best system to be applied for the hydrolysis of biomasses at high WIS concentration. They demonstrated that, at a fixed impeller speed of 10 rpm, the WIS content did not influence the energy input for *Arundo donax*, while a higher overall energy input was required for the hydrolysis of the spruce because, for the latter system, a strong correlation between initial WIS content and energy input was observed. This is explained by a quick drop in torque and viscosity that occurred during the saccharification of *Arundo donax*, as reported for other biomasses (Dasari et al. 2009), and that was much less noticeable for the spruce. In detail, when the impeller speed was kept constant and the WIS concentration was increased from 10 to 20 %, the glucose yield decreased from ~40 to ~27 % for *Arundo donax* and increased from ~20 to ~30 % for the spruce. Otherwise, when the impeller power was kept constant, the glucose yield was equivalent to that obtained at constant impeller speed for *Arundo donax*, while an opposite trend was observed for the spruce, since the glucose yield decreased from ~45 to ~30 % when the WIS content increase from 10 to 20 %. It could be due to a different shear force in the reactor between the two biomasses. Furthermore, Kadić et al. (2014) investigated the effect of agitation rate on the particle-size distribution (PSD) and glucan release during hydrolysis of the steam pretreated *Arundo donax* and spruce. The 2.5-L Biostat A and Biostat A Plus bioreactors (B. Braun Biotech International, Germany), equipped with a **pitched-blade impeller with three blades at an angle of 45 °** (diameter of 70 mm and a blade width of 20 mm) (Fig. 3b), were used for the hydrolysis. Three impeller speeds 100, 300, and 600 rpm were tested, evaluating their different effects on the biomass-particles mixing. In the case of spruce, the effects of agitation rate were only observed using high WIS content (13 %) at higher speed. In fact, no effects during the hydrolysis were observed at 13 % of WIS at 100 rpm, but when the impeller speed was enhanced up to 600 rpm, an increase in the hydrolysis rate from 20 to 37 % after 48 h took place. This could be explained as a result of strong reduction of particle size that improves the sugars released, increasing the hydrolysable surface area. Otherwise, for *Arundo donax* hydrolysis, a smaller particle size than spruce was observed both at low and high impeller speed; in spite of this, only a slight temporary effect (from 43 % at 100 rpm to 53 % at 600 rpm after 48 h) on the hydrolysis rate at high WIS content (13 %) occurred. After 96 h of hydrolysis, the same rate of ~60 % was reached in both systems. To investigate if the reduction of particle size was caused by the enzymes action or by the agitation rate, further experiments in which the lignocellulose biomasses at 13 % of WIS were agitated at high revolutions per minute without the enzymes addition were performed by Kadić et al. (2014). For the spruce, the effects of agitation rate on the particle size was strongly evident; in contrast, the reduction of particle size of *Arundo donax* was more influenced by the enzyme's action than the agitation rate,

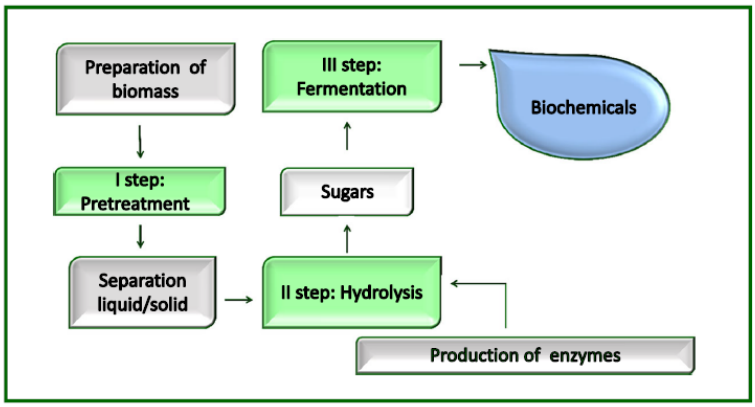

Fig. 1 Main steps of process of lignocellulosic biomass conversion

since the highest size reduction was only observed when the enzymes were loaded. Based on these results, it is important to choose the agitation speed based on the macromolecular structure of the lignocellulosic biomasses and the initial solids loading.

Bioreactors for corn stover conversion

Production of sugars from SHF in batch operation mode

The effect over the yield of sugars obtained during the hydrolysis of the sulfuric acid/steam pretreated corn stover employing two different reactor systems, the horizontal rotating bioreactor (HRR) and the vertical stirred-tank reactor (VSTR), was investigated by Du et al. (2014). The saccharification was performed by using the enzyme Cellic CTec2 from Novozymes, at loading of seven filter paper activity units per gram of dry matter (FPU g^{-1}) at 50 °C in both reactor systems, equipped with thermostatic water bath. The material blending was assured by a mixing blade, at maximum constant rotation speed (100 rpm) in the HRR system and by a **double helical ribbon impeller** (Fig. 3c), at variable speed in the VSTR system. During the hydrolysis, a more rapid viscosity reduction occurred in the HRR, allowing better cellulose degrading in comparison with the VSTR. Comparing the batch and fed-batch (adding biomass or biomass/enzymes) enzymatic hydrolysis, it was demonstrated that the best result of 86 g glucose kg^{-1} of dry matter was obtained after 87 h of incubation in the HRR through the batch hydrolysis in comparison with the value of 73 g glucose kg^{-1} of dry matter shown by the batch VSTR system.

It is well known that the use of ultrasound for lignocellulose treatment improves the enzymatic hydrolysis yield (Khanal et al. 2007; Nitayavardhana et al. 2008; Montalbo-Lomboy et al. 2010a). Recently, Montalbo-Lomboy et al. (2010b) investigated the influence of the batch system over the saccharification sugars yield and the particles size of the corn slurry. The STARGEN™ 001 from Genencor, at loading of 456 granular starch hydrolyzing units per gram of substrate (GSHU g^{-1}), was added after sonication and was used as enzyme in the hydrolysis step for 3 h in a rotary shaker. The results showed a yield of reducing sugars obtained with the batch system equal to 1.6 g L^{-1}.

The high solid processing of corn stover (PCS) represents one of the main drawbacks in the saccharification step. Even though an initial high PCS allows to obtain different advantages, like the reduction of reactor size, it is generally avoided due to the high viscosity shown and the high power required

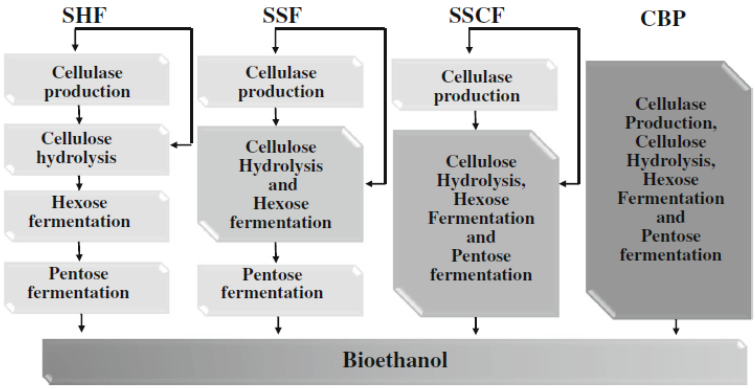

Fig. 2 Processes of sugars and bioethanol production. SHF separate hydrolysis and fermentation, SSF simultaneous hydrolysis and fermentation, SSCF simultaneous saccharification and co-fermentation of both hexoses and pentoses

Table 1 Main characteristics of some examples of reactors for conversion of *Arundo donax*

Biomass	Reactor system	Agitation	Tank volume	Configuration process	Glucose concentration/yield/productivity	Reference
Arundo donax	Stirred tank bioreactor	Anchor impeller	3.0 L	Enzymatic hydrolysis	~40 % of glucose yield at constant impeller speed	Palmqvist and Lidén 2012
Arundo donax	–	Blade impeller with three blades at an angle of 45 °	–	Enzymatic hydrolysis	~60 % of glucose yield	Kadić et al. 2014

to mix homogenously the substrates. Dasari et al. (2009) designed a 8-L scraped surface bioreactor (SSBR), equipped with three scraping blades, to improve the saccharification of the corn stover at high initial PCS. They compared the glucose liberated in the process by using the bioreactor against the results obtained in 250-mL Erlenmeyer flasks. In this work, the saccharification was performed at 50 °C with 15 FPU of Spezyme CP cellulase enzyme (Genencor) per gram of cellulose, at speed of 250 rpm in flasks and 2 rpm in bioreactor. The latter system allowed to reach a glucose yield of 60 %, a value 10 % higher than that obtained in flasks (50 %) and, within the first hours of the hydrolysis reaction, a quick decrease in torque was observed, allowing a low-energy consumption. This was due to the random radial and angular mixing of the biomass and the homogeneous temperature generated by the horizontal rotation and the scraping of the blades.

It is well known that the enzymatic hydrolysis represents the limiting step of the overall costs of lignocellulose bioconversion process. Thus, several kinetic models to reduce both costs and efforts of the step were generated (Philippidis and Hatzis 1997; Gusakov et al. 1985; Sadana 1992; Kadam et al. 2004. One of them was elaborated and validated considering not only the reaction for conversion of cellulose into glucose and cellobiose, but also for the conversion of the cellobiose into glucose (Kadam et al. 2004). Moreover, parameters like enzyme adsorption, substrate reactivity, temperature, and sugar inhibition were taken into account. Following the model, the sulfuric acid-treated corn stover was saccharified with 45 mg protein per gram of cellulose (CPN commercial cellulase, Iogen Corp., Ottawa, Canada) at 45 °C in 250-mL baffled Erlenmeyer flasks stirred at 130 rpm, or in stirred-tank reactor with impeller speed of 250 rpm. The results demonstrated that the model fitted well to predict the glucose yield both in flasks and in tank reactor. The maximum glucose concentration of around 50 g kg^{-1} of cellulose, after 168 h, was obtained for both systems used. Although the temperature effect was not completely defined by the model, this could be exploited to optimize the saccharification process in silico.

Production of sugars from SHF in fed-batch/continuous operation mode

Montalbo-Lomboy et al. (2010b) investigated also the influence of the continuous-flow ultrasonic system over the saccharification sugars yield and the particle size of the corn slurry. Enzyme hydrolysis was performed in an ultrasonic reactor fitted with a donut-shaped horn using the same enzyme amount and process parameters described earlier. In addition, in this system, the corn slurry was localized in the center of the donut-shaped horn where the cavitation was more intense, increasing the liquefaction of the biomass. The results showed an increase up to 2–3 times of the sugars yield in the sonicated

Table 2 Main characteristics of some examples of reactors for conversion of corn stover

Reactor system	Agitation	Tank volume	Configuration process	Glucose concentration/yield/productivity	Reference
Corn stover					
Horizontal rotating bioreactor (HRR) and vertical stirred-tank reactor (VSTR)	Mixing blade in the HRR system and a double helical ribbon impeller in the VSTR system	–	Batch and fed-batch	86 g glucose kg^{-1} of dry matter in the HRR through the batch hydrolysis and 73 g glucose kg^{-1} of dry matter in the batch VSTR system through the batch hydrolysis	Du et al. 2014
8-L scraped surface bioreactor (SSBR)	3 scraping blades	–	Enzymatic hydrolysis	Glucose yield of 60 %	Dasari et al. 2009
Stirred-tank reactor	–	–	Enzymatic hydrolysis	50 g glucose kg^{-1} of cellulose	Kadam et al. 2004
5 bioreactors connected in series for the CSTR process	–	–	SHF and SSCF in batch and continuous mode	Volumetric productivity of 0.25 g L^{-1} h^{-1} and 0.20 g L^{-1} h^{-1} in batch SSCF and SHF, respectively. Maximum volumetric productivity of 0.46 g L^{-1} h^{-1} in continuous SSCF by using a CSTR	Jin et al. 2013
–	Double helical impeller and Rushton impeller	5.0 L	SSF	51.0 g L^{-1} of ethanol in the SSF by using the double helical impeller and 43.9 g L^{-1} in the SSF by using the Rushton impeller	Zhang et al. 2010
	Single helical ribbon impeller		SSF	56.2 g L^{-1} of ethanol	He et al. 2014
Corn slurry					
–	–	3.0 L	Batch cSSF and the 3-stage CSTR cSSF	Ethanol yield of 70 % in the batch and in the 3-stage CSTR compared to the 0.18 g L^{-1} h^{-1} batch one. Maximum productivity of 0.4 g L^{-1} h in the single-vessel CSTR	Brethauer et al. 2014
Ultrasonic reactor equipped with a donut-shaped horn	–	–	Batch and continuous flow	61.6 g L^{-1} in batch system and 30.2 g L^{-1} in continuous flow	Montalbo-Lomboy et al. 2010b

Table 3 Main characteristics of some examples of reactors for conversion of wheat straw

Reactor system	Agitation	Tank volume	Configuration process	Glucose[a] or ethanol[b] concentration/yield/productivity	Reference
Stirred tank reactor	Segmented helical stirrer	–	Enzymatic hydrolysis	[a]Glucose yield of 76 % and 110 g glucose kg^{-1} biomass	Ludwig et al. 2014
48 parallel stirred tank bioreactors	S-shaped impellers	10 mL	Enzymatic hydrolysis	[a]~111 mg glucose g^{-1} dry matter	Riedlberger and Weuster-Botz 2012
–	System of rotating paddle	5.0 L	Batch process, Sequential hydrolysis and solids-recycling processes	[a]Glucose yield of 59 % in batch process, 53 % in sequential hydrolysis, and 63 % in solids-recycling processes. Productivity of 54 and 30 % higher than that reached in the batch system (0.8 g L^{-1} h^{-1}) were observed in the sequential hydrolysis and solids-recycling systems, respectively	Pihlajaniemi et al. 2014
Horizontally placed drum divided into 5 independent sections	Three paddlers	–	8 h of liquefaction and pre-saccharification following by 84 SSF	[b]48 g ethanol kg^{-1} of biomass	Jørgensen et al. 2007b
–	–	2.5 L	Fed-batch SSCF and the enzyme feeding SSCF	[b]0.35 g g^{-1} of ethanol yield and 38 g L^{-1} of the ethanol concentration	Olofsson et al. 2010a

samples in comparison with the control and the yield of reducing sugars obtained was 30.2 g L^{-1}.

Production of ethanol from SSF/CBP in batch operation mode

Brethauer et al. (2014) performed a simultaneous saccharification and fermentation (SSF) of acid-pretreated corn stover in batch system. Five filter paper activity units per gram of glucan of Spezyme CP cellulase (Genencor) and 7.5 CBU g^{-1} of glucan of Novozyme 188 ß-glucosidase (Novozyme) were employed for the saccharification step, while the strain *Saccharomyces cerevisiae* D5A was used for the fermentation at 38 °C. The corn slurry, at low solids concentration, was pumped from the reservoir, equipped with a magnetic stir plate, to the 3-L fermentor together with air bubbles to avoid the accumulation of solid in the tube. They observed an ethanol yield of 70 % and an ethanol productivity of 0.18 g L^{-1} h^{-1}.

Jin et al. (2013) evaluated the conversion of ammonia fiber expansion (AFEX)-pretreated corn stover into ethanol by using two different systems: the SHF and the SSCF. An enzymatic mix containing Accellerase 1500, Accellerase XY, and Multifect pectinase (Genencor) at loadings of 24, 6, and 6 mg g^{-1} of glucan, respectively, was used for the hydrolysis of corn stover. A genetically modified strain of *S. cerevisiae* 424A fermenting xylose was employed for the ethanol fermentation. Firstly, they noted that, although more sugars were released in the SHF and the same ethanol yield was reached in both systems (80 and 47 % of glucose and xylose conversion into ethanol, respectively), the volumetric productivity was 0.25 g $L^{-1}h^{-1}$ for the batch SSCF compared to the 0.20 g $L^{-1}h^{-1}$ of the SHF.

Zhang et al. (2010) designed a reactor with a new agitation system namely double helical impeller (Fig. 3c), in substitution of the common **Rushton impeller** (Fig. 3d), to perform the simultaneous saccharification and fermentation (SSF) of corn stover using high solids loading. Accellerase 1000 from Genencor International (Rochester, NY) at different dosages, and the thermo- and inhibitor-tolerant baker's yeast mutant *S. cerevisiae* DQ1 were used for the saccharification and fermentation steps, respectively. The experiments were conducted in the 5-L bioreactor, performing a prehydrolysis step for 12 h at 50 °C followed by the SSF step at 37 °C to allow the *S. cerevisiae* DQ1 growth. They demonstrated how the new agitation system improved the ethanol yield: 51.0 g L^{-1} of ethanol were obtained at the end of SSF by using the double helical impeller, respect to the 43.9 g L^{-1} reached in the reactor equipped with the Rushton impeller. The best yield was obtained using the double helical impeller due to the better mixing, and this system also reduced the overall process energy demand. Moreover, they reported that at 30 % of solids loading, the ethanol concentration reached 40.0, 59.3, and 64.6 g L^{-1}

Table 4 Main characteristics of some examples of reactors for conversion of different biomass residues

Biomass	Reactor system	Agitation	Tank volume	Configuration process	Ethanol concentration (g L^{-1})-yield (%)-Productivity (g L^{-1} h^{-1})	Reference
Prosopis juliflora (Mesquite)	Stirred tank bioreactor	Rushton impeller	3.0 L	Batch SHF	34.78 g L^{-1} - 0.45 g g^{-1} - 3.16 g L^{-1} h^{-1}	Gupta et al. 2012
Prosopis juliflora (Mesquite)	Stirred tank bioreactor	Rushton impeller	3.0 L	Fed-batch SHF	52.83 g L^{-1} - 0.45 g g^{-1} - 4.40 g L^{-1} h^{-1}	Gupta et al. 2012
Agave bagasse	Mini-bioreactor	Peg-mixer	6×30 mL	SHF	64 g L^{-1}	Caspeta et al. 2014
Citrus peel waste	Limonene removal column-immobilized cell reactor system	–	80 mL	SHF	from 14.4 to 29.5 g L^{-1} (ethanol yields 90.2–93.1 %)	Choi et al. 2015
Sunflower seed hull	Batch culture bioreactor system	Teflon-glass impeller/paddle blade magnetic impeller	0.6 L	SHF	9.66 g L^{-1} and ethanol yield 0.41 g g^{-1}	Okur and Saraçoglu 2006
Switchgrass	Steam-jacketed fermenter	Rushton impeller	50 L	Batch SFF	73 %	Isci et al. 2009
Switchgrass	Steam-jacketed fermenter	Blade axial flow impeller	350 L	Fed-batch SFF	74 %	Isci et al. 2009
Miscanthus	Twin screw reactor-fermenter	Rushton impeller (attached at the cap)	5 L	Fed-batch SFF	74.5 g L^{-1} - 89.5 % - 1.4 g L^{-1} h^{-1}	Han et al. 2014
Poplar sawdust	Continuous twin screw reactor-fermenter	–	4×1 L	Fed-batch SFF	39.9 g L^{-1}	Kim et al. 2013
Cogon grass	Rotary drum reactor	rotation	5 L	SSF	19.1 g^{-1} L^{-1} (yield 76.2 %)	Lin and Lee 2011
Sugarcane bagasse	Rotary drum reactor	rotation	100 L	SSF	24.6 g L^{-1} (yield 79 %)	Lin et al. 2013
Spruce chips	Three-unit integration system (hydrolysis reactor, filtration/pump system, fermentation reactor)	helical stirrer	2.5-L hydrolysis reactor/1.5 L fermentation reactor	SSFF	31.1 g L^{-1} ethanol, corresponding to 85.0 % theoretical yield	Ishola et al. 2013
Poplar wood	Stirred vessel fermentor (Biostat, Sartorius)	Rushton-type stirrer blades	2 L	CBP	34.8 mM (1.06 g L^{-1})	Svetlitchnyi et al. 2013

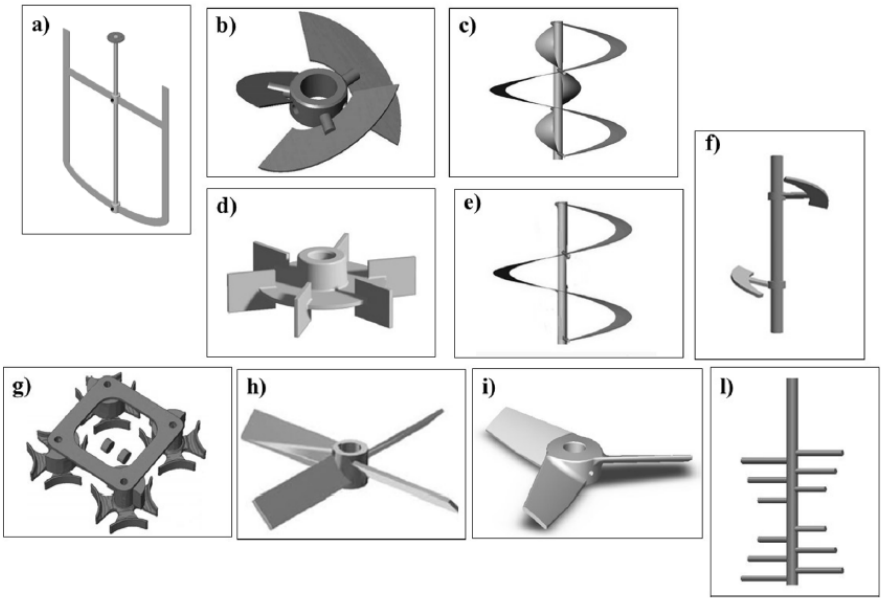

Fig. 3 Agitation systems used in the enzymatic hydrolysis and fermentation of lignocellulosic biomasses. **a)** Anchor impeller. **b)** Blade impeller with three blades at an angle of 45 °. **c)** Double helical ribbon impeller. **d)** Rushton impeller. **e)** Single helical ribbon impeller. **f)** Segmented helical stirrer. **g)** S-shaped impellers. **h)** Rotating paddle. **i)** Three paddlers. **l)** peg mixer

at enzyme dosages of 7.0, 15.0, and 30.0 FPU g^{-1} of dry matter, respectively. Recently, He et al. (2014) performed the dry acid pretreatment of corn stover at high solids concentration by using a reactor equipped with a **single helical ribbon impeller** (Fig. 3e), demonstrating as the steady helically agitation leads to increase sugars and ethanol yields production. The corn stover was treated with 2.5 % H_2SO_4 for 3 min at 185 °C in agitation, followed by inoculation of the strain *Amorphotheca resinae* ZN1 (Chinese General Microorganisms Collection Center, Beijing, China; registration number: CGMCC 7452) to remove specific inhibitor compounds. Afterwards, the biodetoxified corn stover was hydrolyzed with the enzyme Youtell #6 at a loading of 135 FPU g^{-1} of substrate, and fermented by the strain *S. cerevisiae* DQ1(Chinese General Microorganisms Collection Center, Beijing, China; registration number: CGMCC 2528). The SSF consisted of a 12 h of prehydrolysis at 50 °C and pH 4.8, followed by a reduction of temperature at 37 °C to promote the growth of the yeast and its sugars fermentation into ethanol. The results showed that the helical agitation during the pretreatment allows the increment of the sugars released during the hydrolysis, giving 81.9 g L^{-1} of glucose compared to the low value of 55.8 g L^{-1} obtained without mixing. As regards the ethanol production, 56.2 g L^{-1} were obtained after 48 h of fermentation instead of 44.4 g L^{-1} reached when no agitation was employed during the pretreatment.

Production of ethanol from SSF/CBP in fed-batch/continuous operation mode

Very few works about the simultaneous saccharification and fermentation (SSF) of corn stover in a continuous stirred tank reactor (CSTR) are available (Wooley et al. 1999; Jin et al. 2013), due to the limits related to the experimental troubles, although this system increases the volumetric productivity, mostly when more tank reactors are used (Brethauer and Wyman 2010). Brethauer et al. (2014) performed also a continuous simultaneous saccharification and fermentation (cSSF) of acid-pretreated corn stover using a 3-stage CSTR system. Hydrolysis and fermentation were performed in a 3-L fermentor as described earlier. They observed that at the same ethanol yield of 70 %, an ethanol productivity of 0.24 g $L^{-1}h^{-1}$ was observed in the 3-stage continuous system. Moreover, in 8 h of residence time, the single cSSF vessel reached the maximum ethanol productivity of 0.4 g $L^{-1}h^{-1}$, a value that dropped when the total resident time was kept constant and the number of vessels was increased. In other experiment, when the total residence time was 24 h, the productivity increased from 0.25 to 0.28 g $L^{-1}h^{-1}$ when changed from one to three vessels, respectively.

Jin et al. (2013) evaluated the conversion of AFEX pretreated corn stover into ethanol also by continuous SSCF using a CSTR equipped with five bioreactors connected in series. The first reactor was used for the enzymatic hydrolysis at 50 °C, pH 4.8, and 800 rpm; then, the 24-h prehydrolysate was pumped in the next reactor for the SSCF at 30 °C, pH 5.5, and 220 rpm. This system reached the highest volumetric productivity of 0.46 g $L^{-1}h^{-1}$, value 2.3 and 1.8 higher than that observed in the batch SHF and SSCF, respectively. The use of high solids content of lignocellulose biomasses could decrease the loss of sugars, waste of water and steam generation, and also to lead an increment in the rate of bioconversion into ethanol (Kristensen et al. 2009). In spite of this, the

high solids loading is related to operative issues as the high viscosity and the little free water, that limit the pretreatment method which can be performed (Modenbach and Nokes 2012).

Bioreactors for wheat straw conversion

Production of sugars from SHF

Several studies regarding the design of the bioreactor systems to be employed in the saccharification of the wheat straw at high solids loading and, that ensuring an effective mixing and a high bioconversion yield, were so far reported (Jørgensen et al. 2007a; Szijártó et al. 2011a, b). Ludwig et al. (2014) designed a new vertical stirred tank reactor supplied with a **segmented helical stirrer** (Fig. 3f) to hydrolyze the wheat straw, after alkaline-pretreatment, at high solids loading. Before testing the new system, they carried out a central composite response surface analysis to optimize the hydrolysis conditions, minimizing the enzyme dosage and maximizing the fiber concentration. The optimization was carried out in 250-mL Erlenmeyer flasks at 50 °C by using the Cellulase Cellic® CTec2 (Novozymes A/S) as hydrolytic enzyme. After 48 h, a glucose yield of 70 % was reached at the optimal solid concentration of 20 % (*w*/*w*) and an enzyme dosage in the range of 20–30 FPU g^{-1} dry matter. When the hydrolysis was performed in the new stirred reactor, at the same optimized conditions and at a speed of 80 rpm, the glucose yield was increased from 70 to 76 %, corresponding to a hydrolysate with 110 g glucose kg^{-1} biomass. The swelling of the fibers generated by the high hemicellulose content of the wheat straw did not allow exceeding the 20 % (*w*/*w*) of dry matter. Riedlberger and Weuster-Botz (2012) reported an accurate high-throughput system suitable for acid or alkaline-pretreated wheat straw, in order to reduce the costs for the optimization of the enzymatic hydrolysis step related to the large use of the enzymes required. The new system, consisting of 48 parallel stirred-tank bioreactors at volume of 10 mL, was equipped with the novel **S-shaped impellers** (Riedlberger and Weuster-Botz 2010) (Fig. 3g). The homogenization of fibers at high solids loading was achieved by two permanent magnets (IBS Magnet, Berlin, Germany) that drove the rotation of the impeller around a fixed axis. Three different solid contents, 4, 8, and 10 % (*w*/*w*) of pretreated dried wheat straw were saccharified with 15 mg protein g^{-1} dry matter and 1.9 mg protein g^{-1} dry matter of Celluclast® and Novozym® 188 (Novozymes A/S), respectively. After 9-h of hydrolysis, the glucose released (~111 mg g^{-1} dry matter) using the high-throughput system was comparable to the 1-L scale. This test demonstrated the efficient and easy scale-up of the novel system that can be used for the optimization of pretreatment conditions. Other ways to reduce the costs of the saccharification process can be recycling the enzymes or the solid residues, exploiting the ability of adsorption onto lignin and cellulose fractions (Rodrigues et al. 2012; Lee et al. 1994). Pihlajaniemi et al. (2014) investigated the solids-recycling for the hydrolysis of the autohydrolysed wheat straw; moreover, they compared the hydrolysis yield and the volumetric sugars productivity obtained through the solids-recycling for the sequential and the batch reactions, at similar loading of enzymes, substrate, and total liquid. A commercial enzyme mix consisting of cellulase (Econase CE, AB Enzymes), β-glucosidase (Novozyme 188), and xylanase (GC 140, Genencor) was used for the hydrolysis. The reaction used at solids loading of 16 % (*w*/*w*) was carried out in a 5-L reactor composed by a horizontal cylinder and a system of **rotating paddle** (Fig. 3h), at speed rotation of 2 rpm. The hydrolysis yield was almost comparable among the three systems used, whereas differences were observed in the volumetric productivity. Hydrolysis yields of 56 and 59 % were reached in the batch process at 48 and 72 h, respectively. Similar values of 53 % after 48 h and 63 % after 72 h of reaction were obtained for both sequential hydrolysis and solids-recycling processes. Regarding the productivity, 1.4 and 0.8 g L^{-1} h^{-1} were obtained in 48 and 72 h of batch hydrolysis, respectively, using enzyme dose of 9 FPU g^{-1}. Values of 54 and 30 % higher were observed when the hydrolysis was performed in sequential hydrolysis or solids-recycling systems, at 48 and 72 h, respectively. Although the hydrolysis yields were comparable and the productivity values were slightly lower at 72 h than at 48 h of the processes, both the solids-recycling and sequential hydrolysis system could be applied for an efficient enzymatic hydrolysis of the pretreated wheat straw.

Production of ethanol from SSF/CBP in batch operation mode

The use of lignocellulosic biomasses at solids loading above 15 % (*w*/*w*) of dry matter is required in order to obtain an ethanol concentration more than 4 % (*w*/*w*) and thus, making economically feasible the bioconversion process (Fan et al. 2003; Wingren et al. 2003). The high solids loading needs a mixing system to reduce the problematic related to the liquefaction and saccharification steps, like the initial viscosity and the high concentration of the inhibitory compounds. Jørgensen et al. (2007b) designed a reactor system useful for an efficient mixing during the liquefaction and saccharification of the pretreated wheat straw at low speed rates. A reactor consisted of a horizontally placed drum divided into five independently sections equipped by **three paddlers** (Fig. 3i) that were assembled around a horizontal rotating shaft. They investigated the effects over the liquefaction and glucose releasing varying the mixing speed and the initial dry matter content, carrying a liquefaction and saccharification steps for 96-h treatment; moreover, they evaluated the yield of ethanol after 8-h liquefaction and presaccharification followed by 84 h SSF. The enzyme cocktail consisting of

Celluclast 1,5 FG L and Novozym 188 (Novozymes, Denmark) was used for the saccharification The mixing speed range tested was between 3.3 and 11.5 rpm, while the dry matter content went from 20 to 40 %. They observed that, after 24 h of treatment, the wheat straw structure was completely liquefied at low speed of 3.3 rpm. They also reported that the mixing speed did not influence the cellulose conversion in the tested range; differently, the hemicellulose conversion was influenced in a negative way, since it decreased 18 % when the mixing speed increased from 3.3 to 11.5 rpm. Regarding the effect of the dry matter, they obtained a maximum of 86 g glucose kg^{-1} of biomass after 96 h of treatment at solid loading of 40 % (*w*/*w*). As reported in other works (Ingesson et al. 2001; Lu et al. 2002; Tengborg et al. 2001), although the maximum glucose released was reached at the highest initial dry matter, a decrement of the bioconversion yield based on the total initial cellulose content, was observed when the dry matter was increased. When they evaluated the effect of different initial dry matter (from 2 to 40 % (*w*/*w*) over the 84 SSF process, after 8 h of liquefaction and pre-saccharification at 50 °C, the highest ethanol concentration of 48 g kg^{-1} of biomass was obtained at 35 % (*w*/*w*) dry matter after 144 h. According to other experiments (Mohagheghi et al. 1992; Devantier et al. 2005), a drop of the fermentation performance at value of dry matter higher than 35 % (*w*/*w*) was observed, due to the stress conditions (i.e., high osmotic pressure, ethanol, and inhibitor concentration) determining the loss of viability of the yeast.

Production of ethanol from SSF/CBP in fed-batch operation mode

In order to obtain a high yield of ethanol from the bioconversion of the lignocellulosic biomasses, it is necessary to convert all the available sugars, hexoses and pentoses, that are both present in the macromolecular structure. Olofsson et al. (2010a) reported how the SSCF process of the acid-pretreated wheat straw, combining the fed-batch and the enzyme feeding, improves the glucose and xylose co-fermentation of the recombinant xylose-fermenting strain *S. cerevisiae* TMB3400 (Wahlbom et al. 2003). Through the process, the glucose was released at a very low rate, improving the xylose uptake by the yeast (Olofsson et al. 2008, 2010b). A 2.5-L bioreactors (Biostat A. B. Braun Biotech International, Melsungen, Germany; Biostat A plus; Sartorius, Melsungen) was used for the process in anaerobic conditions (Palmqvist et al. 1996). The saccharification of wheat straw was performed by using the enzyme mix consisting of the Xylanase XL (SAF-ISIS, Souston, France) and Novozyme 188 (Novozymes, Denmark). In all the SSCF experiments, the feed of the substrate was performed after 6, 12, 18, and 24 h, starting from the solids loading of 8 % until reaching the value of 11 %. Regarding the enzymes feed, four different profiles, namely A, B, C, and D, were tested; in all cases, a low initial amount of enzyme was added to improve the liquefaction of the substrate. In the profiles A and B, the enzymes were added until 24 and 48 h, respectively; instead, in the profiles C and D, the enzymes were added for the first time during the last addition of the substrate and carried out until 48 h. The profile D differs from C since at 24 h an additional feed of yeast was made, in order to evaluate if a high yield of ethanol could be achieved. As reference experiment, a SSCF in which the feeding of substrates was carried out as described earlier, while the total amount of the enzymes was added at the beginning of the process, was performed. In comparison with the reference experiment, while the other profiles did not gave improvements, profile B gave the best results, allowing an increment from 40 to 50 % of the xylose conversion, from 0.31 to 0.35 g g^{-1} of ethanol yield and from 33 to 38 g L^{-1} of the final ethanol concentration.

Bioreactors for other biomasses conversion

Production of sugars and ethanol from SHF in batch operation mode

Gupta et al. (2012) exploited the saccharification and ethanol production of pretreated mesquite wood in batch experiment. Enzymatic hydrolysis of sodium chlorite-pretreated lignocellulosic biomass was performed in a 3.0-L stirred tank reactor (STR) equipped with Rushton impeller (Fig. 3d) for shaking (150 rpm), heating jacket and heat exchangers for temperature control (50 °C), using 22 FPU g^{-1} of dry substrate (gds) of cellulase (Sigma-Aldrich) and 68 U β-glucosidase/gds (Sigma-Aldrich). Fermentation was performed at 30 °C, a constant speed of 200 rpm, and an aeration of 0.4 vvm, inoculating a *S. cerevisiae* strain to the hydrolyzed slurry after the addition of nutrients (3 g L^{-1} yeast extract, 0.25 g L^{-1} ammonium phosphate dibasic) with an initial pH 6.0. Enzymatic hydrolysis, performed using four different substrate loading values (5, 10, 15, 20 % *w*/*v*) showed that a significant increment in sugars concentration was observed at increasing biomass concentration up to 15 % (from 41.10 to 90.07 g L^{-1}) declining thereafter at the highest substrate level (80.78 g L^{-1} with 40.39 % cellulose conversion). After 11 h of fermentation, a concentration of 34.78±1.10 g L^{-1} (corresponding to a yield of 0.45 g g^{-1} and a productivity of 3.16 g L^{-1} h^{-1}) was reached.

Innovative bioreactors were also developed to use wastes rich in lignocellulosics and residues from industrial and agricultural processes for bioethanol production. Caspeta et al. (2014) developed a system of six units of 30-mL mini-bioreactor (nominal volume) with a **peg-mixer** (Fig. 3l) and a jacked-glass vessel with olives used for water circulating and temperature control during enzymatic hydrolysis, at 50 °C, and sugar fermentations at 37 °C, to improve the SHF process of agave bagasse. Hydrolysis has been conducted using 15 FPU

of Celluclast 1.5 L (NS50013, Novozymes) and 30 CBU of Novozyme 188 (NS50010, Novozymes) per gram of solids, at speed of 150 rpm by a compact overhead stirrer. Using high solids loading (20 %, *w*/*w*), this system enhanced saccharification giving 120 g L^{-1} of glucose, corresponding to 80 % of cellulose conversion after 24 h and a maximum glucose yield of 135 g L^{-1}, corresponding to 90 % cellulose conversion after 64 h. Ethanol production reached values of 64 L^{-1} after 9 h of culture with *S. cerevisiae* strain SuperStart.

Direct conversion of fruit and citrus peel wastes (CPW) into bioethanol, without pretreatment, was investigated by Choi et al. (2015). Hydrolysis of CPW was performed using 12–16 and 10–25 mg protein g^{-1} fruit waste of two enzymes produced in-house from *Aspergillus citrisporus* (Korean Culture Center of Microorganisms KCCM6507) and *Trichoderma longibrachiatum* (Korean Collection for Type Cultures KCTC 6507), in citrate phosphate buffer (pH 4.8) at 45 °C for 48 h at a speed of 180 rpm. Fermentation was conducted in continuous mode at 30 °C in 80-mL immobilized cell reactor (ICR) where *S. cerevisiae* cells was immobilized in alginate drops. A D-limonene removal column (LRC), containing raw cotton and activated carbon, was also joined to the fermentation reactor for desorption of potentially inhibitor substances from the hydrolyzate. About 90 % of CPW enzymatic conversion into fermentable sugars was reported after 48 h. The CPW hydrolyzed was fed into the reactor from the feed stock by peristaltic pump at a flow of 0.08 mL min^{-1}. Fermentation in the LCR-ICR system resulted in high ethanol concentrations reaching values from 14.4 to 29.5 g L^{-1} (ethanol yields 90.2–93.1 %) that were 12-fold higher than ethanol values recovered in the ICR fermentation performed without LCR.

Okur and Saraçoglu (2006) reported that the aeration rate largely effected the ethanol formation from hydrolyzed crop residues in a bioreactor in uncontrolled pH conditions. Acid hydrolysis of sunflower seed hull, performed using a relatively low temperature (90 °C) and low H_2SO_4 concentration (0.7 M), allowed recovering approximately 90 % of sugars from hemicellulose. Detoxified acid hydrolyzed, containing 35–40 g L^{-1} of total reducing sugars, was used for ethanol production with the yeast *Pichia stipitis*. Fermentation was performed in a batch culture bioreactor system consisting of a 0.6-L glass flask with a Teflon and silicone-lined top cap, a Teflon and glass impeller on the top cover, and a paddle blade magnetic impeller (Fig. 3c) on the bottom of flask. The fermentation process was carried out at 30 °C with agitation of 100 rpm and the air was sparged by a flowmeter from the bottom of the vessel at different aeration rates (0, 2.88, 5.76, 7.99 vv^{-1} min^{-1}). Authors reported that oxygen supply stimulated yeast growth and ethanol formation although depending on aeration rate. In fact, the highest sugar consumption (78 %), ethanol concentration (9.66 g L^{-1}) and ethanol yield (0.41 g g^{-1}) was reported at the lowest tested flow rate (2.88 vv^{-1} min^{-1}).

Production of sugars and ethanol from SHF in fed-batch operation mode

SHF fed-batch experiments were performed by Gupta et al. (2012) using pretreated Mesquite wood in comparison with the batch system described earlier. Enzymatic hydrolysis was carried out in a 3.0-L STR as described above with an initial solids concentration of 5 % and adding 11 FPU/gds of cellulose, 34 U β-glucosidase/gds, and 5 % of solids after 24, 56, and 80 h. The use of a fed-batch system resulted in a further increase of sugars production (127 g L^{-1} with 63.56 % cellulose conversion) of 56 % respect to the batch system with a WIS content of 20 %. The highest sugars content resulted also in the highest ethanol concentration in fed-batch process. In fact, ethanol production of 52.83 g L^{-1} (ethanol yield and productivity of 0.45 g g^{-1} and of 4.40 g L^{-1} h^{-1}, respectively) was observed.

Production of ethanol from SSF/CBP in batch operation mode

Switchgrass (*Panicum virgatum* L.) is adopted as a model energy crop by the US Department of Energy due to its high biomass yield, grown in different climate conditions and suitability for marginal land use (Kim et al. 2015); therefore different studies have focused on bioethanol production from this crop. Isci et al. (2009) performed SSF process of ammonia-soaked switchgrass using a 50-L (working volume) pilot-scale bioreactor. Pretreated switchgrass biomass soaked in ammonium hydroxide (containing 48 % cellulose, 23 % hemicellulose, and 22 % Klason Lignin) has been directly used for SSF experiments in a 50-L steam-jacketed fermenter equipped with three Rushton-type impellers (Biostat U-50, Sartorius) (Fig. 3d). SSF was conducted at 35 °C and 130 rpm for 72 h after aseptical addition of yeast inoculum and 77 FPU g^{-1} cellulose of cellulase enzyme (Spezyme CP, Genencor Int.). At the end of the process, authors observed an ethanol yield of 73 %.

Rotary drum reactor represents another interesting strategy to improve the homogenization of pretreated lignocellulosic biomass in SSF. Lin and Lee (2011) used this technology to optimize the SSF process of alkaline-pretreated cogon grass. Pretreated biomass was loaded at a quantity of 1 kg (10 % WIS concentration, *w*/*w*) in a 5-L rotary drum reactor and SSF process has been run using 0.258 mL g^{-1} WIS of enzyme Accellerase 1500 and Ethanol Red dry *S. cerevisiae* yeast (1 g L^{-1} dry yeast) at 37 °C and initial pH of 5.0. The reactor was rotated at 5 rpm for 1 min at 0, 24, 48, and 72 h. An ethanol concentration of 19.1 g L^{-1} has been obtained corresponding to 76.2 % of the theoretical ethanol yield.

These results were confirmed scaling up SSF process in a 100-L rotary drum reactor using alkaline-pretreated sugarcane bagasse (Lin et al. 2013). The reactor was arranged by a double-cone rotary reactor providing a double-wall structure

for controlling temperature by circulating either cold or hot water between the double walls. Moreover, it was equipped with wave-shaped baffles along the inner wall surface of the vessel and with CO_2 outlet port. In this reactor, 10 kg of alkaline-pretreated sugarcane bagasse (WIS concentration of 10 %, *w*/*w*) has been processed by SSF at 42 °C for 72 h by using a commercial cellulase Accellerase 1000 (0.2 mL g^{-1} WIS) and *Kluyveromyces marxianus* var. *marxianus* (0.5 g L^{-1}). The reactor was rotated at 5 rpm for 1 min only at the beginning of the process and every 24 h. After 72 h, 24.6 g L^{-1} of ethanol concentration (79.0 % ethanol yield) were obtained.

A particular reactor system has been set up by Ishola et al. (2013) using simultaneous saccharification, filtration and fermentation (SSFF) process. This system included three integrated units: hydrolysis and fermentation vessels among which fermentation broth was circulated by filtration system. In particular, pretreated spruce chips (SO_2-catalized steam explosion) with 10 % suspended solids (SS) was mixed with 35 FPU g^{-1} SS of the commercial enzyme Cellic® CTec3 (Novozymes) in a 2.5-L reactor (Infors AG107504, Minifors, Switzerland) and pre-hydrolyzed for 24 h at 50 °C, pH 5.0, and agitation of 500 rpm. During SSFF process, hydrolyzed slurry was continuously pumped at flow of 0.8 L min^{-1} in the fermentation vessel (1.5-L bioreactor, Biostat®B plus 8843414 Sartorius, Germany) by a cross-flow membrane and simultaneously, the flow of the liquid of fermentation vessel was inverted to the hydrolysis reactor. Moreover, with the aim to equilibrate the uptake in the fermentation reactor, another peristaltic pump pushed the permeate out of the filter module by increasing flow rate from 1.1 to 2.9 mL min^{-1}. To ensure yeast culture sedimenting, the fermentation bioreactor was equipped with a settler. The SSFF process was conducted for 96 h and 31.1 ± 1.2 g L^{-1} ethanol (theoretical yield of 85.0 %) were reached.

Svetlitchnyi et al. (2013) used consolidated bioprocessing (CBP) approach for producing ethanol from poplar wood through thermophilic bacteria without the need for additional cellulolytic enzymes. In particular, washed and unwashed solid fraction of poplar wood obtained after dilute sulfurous acid steam explosion were loaded in 2-L stirred vessel fermentor (Biostat B-DCU, B. Braun/Sartorius AG) equipped with double jackets for temperature control, two Rushton type stirrer blades (Fig. 3d), pH control loops and high-precision blow-off valves for pressure controlling in a range of 1.3–1.5 bar. CBP process was conducted at a constant pH of 6.75 and temperature of 72 °C, inoculating the cellulolytic/xylanolytic strain *Caldicellulosiruptor* sp. DIB 004C (GenBank accession number JX988415) and the fermenting thermophilic ethanologenic/xylanolytic strain *Thermoanaerobacter* DIB 097X (GenBank accession number JX988424) in monocultures and in dual co-cultures. Authors reported that the CBP approach with operating temperatures above 70 °C and developing co-cultures of these bacterial strains led to an efficiently conversion of C6- and C5-sugars from pretreated lignocellulosic material into ethanol (up to 34.8 mM) and other products (33.6 mM) such as lactate and acetate.

Production of ethanol from SSF in fed-batch operation mode

Isci et al. (2009) scaled up SSF process of ammonia-soaked switchgrass using a 350-L steam-jacketed fermenter equipped with three-blade axial flow impeller (Model PTT, Walker Stainless Equipment Co.). The process was carried out at 200 rpm for 120 h in semiaseptic and fed-batch conditions, adding pretreated ammonia-soaked switchgrass biomass at three times (0, 5, and 24 h) to allow the thinning of substrate by cellulase (77 FPU g^{-1} cellulose). Controlling bacterial contamination during the process and improving stirring conditions of pretreated lignocellulosic biomass, it is possible to enhance bioethanol production ensuring the success of SSF scale-up. In fact, authors reported that in the best case in 350-L fermenter the ethanol yield was 74 %, similar to that obtained in 50-L fermenter (ethanol yield 73 %).

Han et al. (2014) developed SSF reactor to generate high-concentration bioethanol from *Miscanthus* biomass. They used a continuous twin-screw extruder for pretreating lignocellulosic biomass. The pretreatment reactor was fed with *Miscanthus* biomass at a rate of 18 g min^{-1} and pretreatment solution at 90 mL min^{-1}. During this process, performed using a deficient amount of NaOH at 95 °C with a rotation of 80 rpm, solid and liquid components were separated using an oil press to reuse the solution obtained after pretreatment. This approach allowed reducing the costs of pretreatment process minimizing wastewater and reducing the amount of expensive alkali catalysts used. The resulting pretreated biomass was continuously fed at a rate of 80–150 g h^{-1} into the bottom of a 5-L tank reactor for SSF until to achieve a final concentration of approximately 25 % (*w*/*v*), containing a glucose concentration of 40 g L^{-1}. SSF process has been conducted at 32 °C with agitation (90 rpm) for 96 h using an enzymatic loading of 30 FPU g^{-1} cellulose (Cellic® CTec2, Novozymes), 15 % Cellic® HTec2 (Novozymes) and 7 % (*v*/*v*) *S. cerevisiae* CHY 1011. The optimized pretreatment process coupled to a fed-batch approach increased the efficiency of hydrolytic enzymes obtaining ethanol at high concentration (up to 74.5 g L^{-1} with a yield and productivity of 89.5 % and 1.4 g L^{-1} h^{-1}) using high solid loadings lead to a reduction of distillation energy costs.

Similar approach was also assayed by Kim et al. (2013) that used a continuous twin screw-driven reactor (CTSR) pretreatment associated to a fed-batch SSF for bioethanol production from poplar sawdust. The reactor was composed of 30 segments for continuous biomass rotation, pulverization, and pressure. In this case, diluted H_2SO_4 (4 %) was used as catalyst in the pretreatment process conducted at 180 °C with a screw rotation speed of 60 rpm and biomass feeding rate of

1 g min^{-1}. Fed-batch SSF experiments were conducted using 30 FPU of Celluclast 1.5 L (Novozymes) and 70 pNPG Novozyme 188 (Novozymes) per gram of cellulose in a bioreactor composed of four units of 1 L in which 6.0 wt % pretreated biomass was added at three stages, maintaining a constant solids concentration. This approach showed a great potential since high ethanol concentration (39.9 g L^{-1}) was achieved.

Conclusions

This review gives an overview of the last advances in the bioreactor configurations used for the conversion of dedicated energy crops and residual materials, describing how parameters like high solids loading, particles size, enzymes recycling, speed/power input, volume, and substrates reactivity, can improve the sugars release and the ethanol concentration. In the last decade, due to the complexity of the lignocellulose macromolecular structure, new bioreactor configurations have been designed and/or applied in order to make feasible the use of high substrate loading during the bioconversion process. Bioreactors equipped with new agitation systems like a special segmented helical stirrer (Ludwig et al. 2014), the S-shaped impellers (Riedlberger and Weuster-Botz 2012) and the double helical ribbon impeller (Du et al. 2014) were constructed to achieve an efficient fiber homogenization, reducing the required energy in conditions of high substrate loading. These agitation configurations enhance the homogeneous mixing of the biomass counteracting the elevate initial viscosity, due to the high biomass dosage, and allowing to profit several advantages related to this condition. As a matter of fact, a high substrate loading can lead to several economic and operative advantages such as the reduction of reactor size, the decrease in the sugars loss and wastes generation and easier downstream processing, due to higher product concentration. However, further developments in the bioreactor configuration combined to new efficient agitation systems and optimal operative conditions are needed to apply the process in pilot or industrial scale and to achieve a high bioconversion yield.

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References

Amore A, Pepe O, Ventorino V, Birolo L, Giangrande C, Faraco V (2012) Cloning and recombinant expression of a cellulase from the cellulolytic strain *Streptomyces sp.* G12 isolated from compost. Microb Cell Fact 11:164–178. doi:10.1186/1475-2859-11-164

Amore A, Pepe O, Ventorino V, Birolo L, Giangrande C, Faraco V (2013) Industrial waste based compost as a source of novel cellulolytic strains and enzymes. FEMS Microbiol Lett 339(2):93–101. doi:10.1111/1574-6968.12057

Amore A, Parameswaran B, Kumar R, Birolo L, Vinciguerra R, Marcolongo L, Ionata E, La Cara F, Pandey A, Faraco V (2015) Application of a new xylanase activity from *Bacillus amyloliquefaciens* XR44A in brewer's spent grain saccharification. J Chem Technol Biotechnol 90:573–581. doi:10.1002/jctb.4589

Brethauer S, Studer MH, Wyman CE (2014) Application of a slurry feeder to 1 and 3 stage continuous simultaneous saccharification and fermentation of dilute acid pretreated corn stover. Bioresour Technol 170:470–476. doi:10.1016/j.biortech.2014.07.049

Brethauer S, Wyman CE (2010) Review: continuous hydrolysis and fermentation for cellulosic ethanol production. Bioresour Technol 101:4862–4874. doi:10.1016/j.biortech.2009.11.009

Caspeta L, Caro-Bermúdez MA, Ponce-Noyola T, Martinez A (2014) Enzymatic hydrolysis at high-solids loadings for the conversion of agave bagasse to fuel ethanol. Appl Energy 113:277–286. doi:10.1016/j.apenergy.2013.07.036

Choi IS, Lee YG, Khanal SK, Park BJ, Bae H-J (2015) A low-energy, cost-effective approach to fruit and citrus peel waste processing for bioethanol production. Appl Energy 140:65–74. doi:10.1016/j.apenergy.2014.11.070

Dasari RK, Dunaway K, Berson RE (2009) A scraped surface bioreactor for enzymatic saccharification of pretreated corn stover slurries. Energy Fuel 23:492–497. doi:10.1021/ef800434u CCC: $40.75

Devantier R, Pedersen S, Olsson L (2005) Characterization of very high gravity ethanol fermentation of corn mash. Effect of glucoamylase dosage, pre-saccharification and yeast strain. Appl Microbiol Biotechnol 68:622–629. doi:10.1007/s00253-005-1902-9

Du J, Zhang F, Li Y, Zhang H, Liang J, Zheng H, Huang H (2014) Enzymatic liquefaction and saccharification of pretreated corn stover at high-solids concentrations in a horizontal rotating bioreactor. Bioprocess Biosyst Eng 37:173–181. doi:10.1007/s00449-013-0983-6

Fagnano M, Impagliazzo A, Mori M, Fiorentino N (2015) Agronomic and environmental impacts of giant reed (*Arundo donax* L.): results from a long-term field experiment in hilly areas subject to soil erosion. Bioenerg Res 8:415–422. doi:10.1007/s12155-014-9532-7

Fan ZL, South C, Lyford K, Munsie J, van Walsum P, Lynd LR (2003) Conversion of paper sludge to ethanol in a semicontinuous solids-fed reactor. Bioprocess Biosyst Eng 26:93–101. doi:10.1007/s00449-003-0337-x

Giacobbe S, Vincent F, Faraco V (2014) Development of an improved variant of GH51 α-L-arabinofuranosidase from *Pleurotus ostreatus* by directed evolution. New Biotechnol 31(3):230–236. doi:10.1016/j.nbt.2014.02.004

Gupta R, Kumar S, Gomes J, Kuhad RC (2012) Kinetic study of batch and fed-batch enzymatic saccharification of pretreated substrate and subsequent fermentation to ethanol. Biotechnol Biofuels 20:5–16. doi:10.1186/1754-6834-5-16

Gusakov AV, Sinitsyn AP, Klyosov AA (1985) Kinetics of the enzymatic hydrolysis of cellulose: 1. A mathematical model for a batch reactor process. Enzyme Microb Technol 7:346–352. doi:10.1016/0141-0229(85)90114-0

Han M, Moon SK, Choi GW (2014) Pretreatment solution recycling and high-concentration output for economical production of bioethanol. Bioprocess Biosyst Eng 37:2205–2213. doi:10.1007/s00449-014-1198-1

He Y, Zhang L, Zhang J, Bao J (2014) Helically agitated mixing in dry dilute acid pretreatment enhances the bioconversion of corn stover into ethanol. Biotechnol Biofuels 7:1–13. doi:10.1186/1754-6834-7-1

Ingesson H, Zacchi G, Yang B, Esteghlalian AR, Saddler JN (2001) The effect of shaking regime on the rate and extent of enzymatic hydrolysis of cellulose. J Biotechnol 88:177–182. doi:10.1016/S0168-1656(01)00273-5

Iqbal HMN, Kyazze G, Keshavarz T (2013) Advances in the valorization of lignocellulosic materials by biotechnology: an overview. Biores 8(2):3157–3176

Isci A, Himmelsbach JN, Strohl J, Pometto AL III, Raman DR, Ane RP (2009) Pilot-scale fermentation of aqueous-ammonia-soaked switchgrass. Appl Biochem Biotechnol 157:453–462. doi:10.1007/s12010-008-8235-y

Ishola MM, Jahandideh A, Haidarian B, Brandberg T, Taherzadeh MJ (2013) Simultaneous saccharification, filtration and fermentation (SSFF): a novel method for bioethanol production from lignocellulosic biomass. Bioresour Technol 133:68–73. doi:10.1016/j.biortech.2013.01.130

Jin M, Gunawan C, Balan V, Yu X, Dale BE (2013) Continuous SSCF of AFEXTM pretreated corn stover for enhanced ethanol productivity using commercial enzymes and *Saccharomyces cerevisiae* 424A (LNH-ST). Biotechnol Bioeng 110(5):1302–1311. doi:10.1002/bit.24797

Jørgensen H, Kristensen JB, Felby C (2007a) Enzymatic conversion of lignocellulose into fermentable sugars: challenges and opportunities. Biofuel Bioprod Bioref 1:119–134. doi:10.1002/bbb.4

Jørgensen H, Vibe-Pedersen J, Larsen J, Felby C (2007b) Liquefaction of lignocellulose at high-solids concentrations. Biotechnol Bioeng 96(5):862–870. doi:10.1002/bit.21115

Kadam KL, Rydholm EC, McMillan JD (2004) Development and validation of a kinetic model for enzymatic saccharification of lignocellulosic biomass. Biotechnol Prog 20:698–705. doi:10.1021/bp034316x CCC: $27.50

Kadić A, Palmqvist B, Lidén G (2014) Effects of agitation on particle-size distribution and enzymatic hydrolysis of pretreated spruce and giant reed. Biotechnol Biofuels 7:77–86. doi:10.1186/1754-6834-7-77

Kajaste R (2014) Chemicals from biomass—managing greenhouse gas emissions in biorefinery production chains—a review. J Clean Prod 75:1–10. doi:10.1016/j.jclepro.2014.03.070

Khanal SK, Montalbo M, van Leeuwen J, Srinivasan G, Grewell D (2007) Ultrasound enhanced glucose release from corn in ethanol plants. Biotechnol Bioeng 98:978–985. doi:10.1002/bit.21497

Khoo HH (2015) Review of bio-conversion pathways of lignocellulose-to-ethanol: sustainability assessment based on land footprint projections. Renew Sust Energ Rev 46:100–119. doi:10.1016/j.rser.2015.02.027

Kim SM, Guo J, Kwak S, Jin YS, Lee DK, Singh V (2015) Effects of genetic variation and growing condition of prairie cordgrass on feedstock composition and ethanol yield. Bioresour Technol 183:70–77. doi:10.1016/j.biortech.2015.02.020

Kim TH, Choi CH, Oh KK (2013) Bioconversion of sawdust into ethanol using dilute sulfuric acid-assisted continuous twin screw-driven reactor pretreatment and fed-batch simultaneous saccharification and fermentation. Bioresour Technol 130:306–313. doi:10.1016/j.biortech.2012.11.125

Kristensen JB, Felby C, Jorgensen H (2009) Yield-determining factors in high-solids enzymatic hydrolysis of lignocellulose. Biotechnol Biofuels 2(1):11–20. doi:10.1186/1754-6834-2-11

Lee D, Yu AHC, Saddler JN (1994) Evaluation of cellulase recycling strategies for the hydrolysis of lignocellulosic substrates. Biotechnol Bioeng 45:328–336. doi:10.1002/bit.260450407

Liguori R, Amore A, Faraco V (2013) Waste valorization by biotechnological conversion into added value products. Appl Microbiol Biotechnol 97:6129–6147. doi:10.1007/s00253-013-5014-7

Liguori R, Soccol CR, de Souza Vandenberghe LP, Woiciechowski AL, Faraco V (2015) Second generation ethanol production from brewers' spent grain. Energies 8:2575–2586. doi:10.3390/en8042575

Limayem A, Ricke SC (2012) Lignocellulosic biomass for bioethanol production: current perspectives, potential issues and future prospects. Prog Energ Combust 38:449–467. doi:10.1016/j.pecs.2012.03.002

Lin YS, Lee WC (2011) Simultaneous saccharification and fermentation of alkaline pretreated cogongrass for bioethanol production. BioResources 6:2744–2756

Lin YS, Lee WC, Duan KJ, Lin YH (2013) Ethanol production by simultaneous saccharification and fermentation in rotary drum reactor using thermotolerant *Kluyveromyces marxianus*. Appl Energy 105:389–394

Lu Y, Yang B, Gregg D, Saddler JN, Mansfield SD (2002) Cellulase adsorption and an evaluation of enzyme recycle during hydrolysis of steam exploded softwood residues. Appl Biochem Biotechnol 98(100):641–654. doi:10.1385/ABAB:98-100:1-9:641

Ludwig D, Michael B, Hirth T, Rupp S, Zibek S (2014) High solids enzymatic hydrolysis of pretreated lignocellulosic materials with a powerful stirrer concept. Appl Biochem Biotechnol 172:1699–1713. doi:10.1007/s12010-013-0607-2

Modenbach AA, Nokes SE (2012) The use of high-solids loadings in biomass pretreatment—a review. Biotechnol Bioeng 109:1430–1442. doi:10.1002/bit.24464

Mohagheghi A, Tucker M, Grohmann K, Wyman C (1992) High solids simultaneous saccharification and fermentation of pretreated wheat straw to ethanol. Appl Biochem Biotechnol 33:67–81. doi:10.1007/BF02950778

Montalbo-Lomboy M, Johnson L, Khanal SK, van Leeuwen J, Grewell D (2010a) Sonication of sugary-2 corn: a potential pretreatment to enhance sugar release. Bioresour Technol 101(1):351–358. doi:10.1016/j.biortech.2009.07.075

Montalbo-Lomboy M, Khanal SK, van Leeuwen JH, Raman DR, Dunn L, Grewell D (2010b) Ultrasonic pretreatment of corn slurry for saccharification: a comparison of batch and continuous systems. Ultrason Sonochem 17:939–946. doi:10.1016/j.ultsonch.2010.01.013

Nitayavardhana S, Rakshit SK, van Leeuwen J, Grewell D, Khanal SK (2008) Ultrasound pretreatment of cassava chip slurry to enhance sugar release for subsequent ethanol production. Biotechnol Bioeng 101(3):487–496. doi:10.1002/bit.21922

Okur MT, Saraçoglu NE (2006) Ethanol production from sunflower seed hull hydrolysate by *Pichia stipitis* under uncontrolled pH conditions in a bioreactor. Turkish J Eng Env Sci 30:317–322

Olofsson K, Palmqvist B, Lidén G (2010a) Improving simultaneous saccharification and co-fermentation of pretreated wheat straw using both enzyme and substrate feeding. Biotechnol Biofuels 3:17–25. doi:10.1186/1754-6834-3-17

Olofsson K, Rudolf A, Lidén G (2008) Designing simultaneous saccharification and fermentation for improved xylose conversion by a recombinant strain of *Saccharomyces cerevisiae*. J Biotechnol 134: 112–120. doi:10.1016/j.jbiotec.2008.01.004

Olofsson K, Wiman M, Lidén G (2010b) Controlled feeding of cellulases improves conversion of xylose in simultaneous saccharification and cofermentation for bioethanol production. J Biotechnol 145:168–175. doi:10.1016/j.jbiotec.2009.11.001

Palmqvist B, Lidén G (2012) Torque measurements reveal large process differences between materials during high solid enzymatic hydrolysis of pretreated lignocellulose. Biotechnol Biofuels 5:57–65. doi:10.1186/1754-6834-5-57

Palmqvist E, Hahn-Hägerdal B, Galbe M, Larsson M, Stenberg K, Szengyel Z, Tengborg C, Zacchi G (1996) Design and operation of a bench-scale process development unit for the production of ethanol from lignocellulosics. Biores Technol 58:171–179. doi:10.1016/S0960-8524(96)00096-X

Philippidis GP, Hatzis C (1997) Biochemical engineering analysis of critical process factors in the biomass-to-ethanol technology. Biotechnology Prog 13:222–231. doi:10.1021/bp970017u

Pihlajaniemi V, Sipponen S, Sipponen MH, Pastinen O, Laakso S (2014) Enzymatic saccharification of pretreated wheat straw: comparison of solids-recycling, sequential hydrolysis and batch hydrolysis. Biores Technol 153:15–22. doi:10.1016/j.biortech.2013.11.060

Popp J, Lakner Z, Harangi-Rákos M, Fári M (2014) The effect of bioenergy expansion: food, energy, and environment. Renew Sust Energ Rev 32:559–578. doi:10.1016/j.rser.2014.01.056

Riedlberger P, Weuster-Botz D (2010). Rührorgan für Flüssigkeiten. German Patent Application DE 20 2010 011 902 U1

Riedlberger P, Weuster-Botz D (2012) New miniature stirred-tank bioreactors for parallel study of enzymatic biomass hydrolysis. Biores Technol 106:138–146. doi:10.1016/j.biortech.2011.12.019

Rodrigues AC, Leitão AF, Moreira S, Felby C, Gama M (2012) Recycling of cellulases in lignocellulosic hydrolysates using alkaline elution. Bioresour Technol 110:526–533. doi:10.1016/j.biortech.2012.01.140

Sadana A (1992) Models of enzyme deactivation. In: Gupta MN (ed) Thermostability of enzymes. Narosa, New Delhi, pp 84–93

Salmon DNX, Rigon Spier M, Soccol CR, de Souza Vandenberghe LP, Weingartner Montibeller V, Jucoski Bier MC, Faraco V (2014) Analysis of inducers of xylanase and cellulose activities production by *Ganoderma applanatum* LPB MR-56. Fungal Biol 118:655–662. doi:10.1016/j.funbio.2014.04.003

Scheidel A, Sorman AH (2012) Energy transitions and the global land rush: ultimate drivers and persistent consequences. Global Environ Chang 22:588–595. doi:10.1016/j.gloenvcha.2011.12.005

Shahsavarani H, Hasegawa D, Yokota D, Sugiyama M, KaneKo Y, Boonchird C, Marashima S (2013) Enhances bio-ethanol production from cellulose materials by semi-simultaneous saccharification and fermentation using high temperature resistant *Saccharomyces cerevisiae* TJ14. J Biosci Bioeng 115:20–23. doi:10.1016/j.jbiosc.2012.07.018

Svetlitchnyi VA, Kensch O, Falkenhan DA, Korseska SG, Lippert N, Prinz M, Sassi J, Schickor A, Curvers S (2013) Single-step ethanol production from lignocellulose using novel extremely thermophilic bacteria. Biotechnol Biofuels 6:31. doi:10.1186/1754-6834-6-31

Szijártó N, Horan E, Zhang J, Puranen T, Siika-aho M, Viikari L (2011a) Thermostable endoglucanases in the liquefaction of hydrothermally pretreated wheat straw. Biotechnol Biofuels 4:2–11. doi:10.1186/1754-6834-4-2

Szijártó N, Siika-Aho M, Sontag-Strohm T, Viikari L (2011b) Liquefaction of hydrothermally pretreated wheat straw at high-solids content by purified *Trichoderma enzymes*. Bioresour Technol 102:1968–1974. doi:10.1016/j.biortech.2010.09.012

Tengborg C, Galbe M, Zacchi G (2001) Influence of enzyme loading and physical parameters on the enzymatic hydrolysis of steam-pretreated softwood. Biotechnol Prog 17: 110–117. doi: 10.1021/bp000145+ CCC: $20.00

Ventorino V, Aliberti A, Faraco V, Robertiello A, Giacobbe S, Ercolini D, Amore A, Fagnano M, Pepe O (2015) Exploring the microbiota dynamics related to vegetable biomasses degradation and study of lignocellulose-degrading bacteria for industrial biotechnological application. Sci Rep 5:8161–8174. doi:10.1038/srep08161

Wahlbom CF, van Zyl WH, Jonsson LJ, Hahn-Hägerdal B, Otero RRC (2003) Generation of the improved recombinant xylose-utilizing *Saccharomyces cerevisiae* TMB 3400 by random mutagenesis and physiological comparison with *Pichia stipitis* CBS 6054. FEMS Yeast Res 3:319–326. doi:10.1016/S1567-1356(02)00206-4

Wang Z, Xu J, Cheng JJ (2011) Modeling biochemical conversion of lignocellulosic materials for sugar production: a review. Biores 6(4):5282–5306

Mezule WL, Dalecka B, Juhna T (2015) Fermentable sugar production from lignocellulosic. Chem Eng Trans 43:619–624. doi:10.3303/CET1543104

Weingartner Montibeller V, de Souza Vandenberghe LP, Amore A, Soccol CR, Birolo L, Vinciguerra R, Salmon DNX, Rigon Spier M, Faraco V (2014) Characterization of hemicellulolytic enzymes produced by *Aspergillus niger* NRRL 328 under solid state fermentation on soybean husks. BioRes 9(4):7128–7140

Wingren A, Galbe M, Zacchi G (2003) Techno-economic evaluation of producing ethanol from softwood: comparison of SSF and SHF and identification of bottlenecks. Biotechnol Prog 19:1109–1117. doi:10.1021/bp0340180

Wooley R, Ruth M, Sheehan J, Ibsen K, Majdeski H, Galvez A (1999) Lignocellulosic biomass to ethanol process design and economics utilizing cocurrent dilute acid prehydrolysis and enzymatic hydrolysis: current and futuristic scenarios, National Renewable Energy Laboratory, Golden, CO, NREL/TP-580-26157

Zhang J, Chu D, Huang J, Yu Z, Dai G, Bao J (2010) Simultaneous saccharification and ethanol fermentation at high corn stover solids loading in a helical stirring bioreactor. Biotechnol Bioeng 105(4): 718–728. doi:10.1002/bit.22593

Biological treatment processes for advancing waste biorefinery and advocating circular economy.

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Abstract

The actualization of a circular economy through the use of lignocellulosic wastes as renewable resources can lead to reduce the dependence from fossil-based resources and contribute to a sustainable waste management. The integrated biorefineries, exploiting the overall lignocellulosic waste components to generate fuels, chemicals and energy, are the pillar of the circular economy. The biological treatment is receiving great attention for the biorefinery development since it is considered an eco-friendly alternative to the physico-chemical strategies to increase the biobased products recovery from wastes and improve saccarification and fermentation yields. This paper reviews the last advances in the biological treatments aimed at upgrading lignocellulosic wastes, implementing the biorefinery concept and advocating circular economy.

Keywords:

Microbial delignification; Fungal detoxification; Lignocellulolytic enzymes; Estherases; Hydrolases.

Highlights

- Circular economy is based on renewable resources and sustainable waste management.
- Biological delignification by microbial and enzymatic treatment are described.
- Enzymatic treatment of food, paper, cotton and oil wastes upgrading are reported.
- Further application of fungi in detoxification and enzymes production are reviewed.

1. Introduction

Minimizing the dependence on fossil-based resources and reducing the generation of solid and liquid wastes represent the main challenges for the modern society. The worldwide wastes production was estimated, in the 2010, to be between 7 and 10 billion tonnes, half of which are produced by the most industrialized and developed countries (UNEP, 2015). In spite of this, the globalisation is causing an increment of waste per capita also in non developed countries, moving people from rural to urban areas and increasing the rate of hazardous and industrial wastes (UNEP, 2015).
Most of the worldwide wastes are intended for landfill, or, in the worst cases, for uncontrolled disposal, causing environmental damages and financial loss. It was, indeed, calculated that the costs for the inaction in waste management are 5-10 times more than the actualization of a proper management, due to side effects like tourism crisis and high cost for clean-up the environments (Wilson and Velis, 2015).

Therefore, there is an urgent need for the development of a sustainable waste management aimed at reducing wastes and improving the human and environment health. A beneficial waste management is founded on the realization of a circular economy, in which an integration between the use of renewable resources and the wastes prevention is viable (Allesch and Brunner, 2014).
After the first oil crisis, the first (or rural) and the second (or urban) generation biorefineries have undergone a considerable development, contributing to the increase of the renewable resources usage (Jacquet et al. 2015). In 2013, the renewable energy/resource supplied a 19.1 % of the global energy request, and furthermore, this contribution is continued to expand in 2014 (REN21, 2015).
Lignocellulosic biomasses - including pulp and paper, agriculture, food, forestry and municipal solid wastes - are considered as the major promising renewable resources, since they are largely available around the world and allow to avoid the conflict food *versus* fuel related to the use of edible crops (Gavrilescu, 2014).
A full recycling and re-use of agro-industrial lignocellulosic wastes take place in the second generation biorefinery platform, where bio-based products (food, feed, chemicals) and bioenergy (biofuels, power and heat) are generated through integrated and sustainable processes (Liguori et al. 2013; FitzPatrick et al. 2010; Amore et al. 2014). Circular economy, based on the biorefinery platform and the approach 3Rs - reduce, reuse, recycling of waste, aims at accomplishing a closed-loop system to maximize the recovery of raw materials derived from the waste at end-of-life. The wastes should be considered "renewable resources" that can be used again and again to generate valuable and marketable products, replacing the exhaustible fossil-based resources (Velis, 2015).
This review summarizes the last advances in the biological processes for lignocellulosic wastes upgrading, focusing the attention on the closed loop approach promoted by the circular economy and realized through the biorefinery platform. A schematic overview of the biological treatments and the corresponding upgraded products described in this review is shown in Figure 1. In detail, an overview of delignification and valorization of different lignocellulosic wastes through fungal, bacterial and enzymatic treatments is given, with focus on the advantages and disadvantages related to each method, as listed in Table 1, and on the large spectrum of upgraded products generated by the processes, as reported in Table 2.

2. Advanced biological treatment of lignocellulosic wastes

2.1 Fungal treatment

The last advances in the treatment of agro-industrial wastes through fungal solid state fermentation (SSF) are hereby reported, with focus on the sustainability of the process and the achievable added-value bioproducts. The SSF represents an environmentally and economically friendly alternative to the fisico-chemical ones, since it does not require chemical agents and involves low temperatures and pressures (Chandel et al. 2015; Moreno e al. 2015) (Table 1).
Several studies have been focused on the SSF as a pretreatment step in the biorefinery platform, for delignifying and/or upgrading lignocellulosic biomasses (Khuong et al 2014; Zhao J. et al. 2014; Zhao et al. 2012; Zhao et al. 2013) as well as for detoxifying the lignocellulosic hydrolysates from the products, generated by the hemicellulose and lignin degradation, that reduce the fermentation efficiency (Pereira et al. 2012). Moreover, the fungal SSF has been also largely exploited to produce (hemi)cellulolytic and lignin-degrading enzymes using cheap raw materials as

substrates in order to reduce enzyme production costs (Mutschlechner et al. 2015; Mahajen et al. 2014; Chatzifragkou et al. 2014).
Regarding the exploitation of the SSF for the removal of toxic compounds with the aim to improve the fermentation efficiency, Pereira et al. (2012) demonstrated the potential of the fungus *Paecilomyces variotii* for the detoxification of the hardwood spent sulphite liquor (HSSL) from different microbial inhibitors like acetic acid, furfural and polyphenols. The biological treatment, followed by the *Pichia stipitis* fermentation allowed to reach an ethanol concentration of 2.4 g L^{-1} with a yield of 0.24 g ethanol g^{-1} sugars.

Table 1. Advantages and disadvantages of different biological waste treatment.

Biological waste treatment	Advantages	Disadvantages
Fungal treatment **Bacterial treatment**	• Low cost • Eco-friendly • Low temperature and pressure • No water and chemical agents requirement • No growth inhibitor formation • Low waste generation • Low energy demand	• Long time (about 3–5 months) • Feedstock loss • Low conversion efficiency • No-byproducts tolerant strains
Enzymatic treatment	• Eco-friendly • Low temperature and pressure • Wide temperature and pH ranges • No nutrient supplementation • No sugars consumption • Short time (hours) • High conversion efficiency • Low waste generation • Low energy demand	• High enzyme costs • Moderate enzymatic performances • Enzymes recycling • Enzymes immobilization • Enzymes activity improvement.

In the last decade, many efforts have being focused on the SSF operative parameters optimization in order to maximize the delignification rate and the yield of different added-value bioproducts (Melikoglua et al. 2013; Gujarathia et al. 2015). Concerning the application of SSF as biological delignification process, Khuong et al. (2014) demonstrated that the initial moisture content and the addition of inorganic chemical factors affected delignification rate of the sugarcane bagasse and the following ethanol production carried out by the fungal strain *Phlebia* sp. MG-60. In detail, they showed as the addition of Fe^{2+}, Mn^{2+} and Cu^{2+} at the beginning of the integrated fermentation process and an initial moisture content of 75 % promoted a selective lignin degradation, allowing to reach the 44 % of the effective ethanol yield.
Moreover, fungal treatment was also investigated as pretreatment for the production of biogas – such as methane and hydrogen – as reported in Table 2 (Zhao J. et al. 2014; Zhao et al. 2012; Zhao et al. 2013). Zhao J. et al. (2014) verified the feasibility of methane production through the fungal pretreatment of unsterilized yard

trimmings, thus also avoiding the sterilization that is not economically sustainable in the frame of the biorefinery industries (Salvachúa et al. 2011). In detail, they performed a pretreatment by using *Ceriporiopsis subvermispora* pre-colonized yard trimmings, followed by solid state anaerobic digestion with an effluent collected from a mesophilic liquid anaerobic digester. After 30 days of fungal treatment, 14.8-20.2 % of lignin degradation was reached, and a methane yield of 34.9-44.6 L kg^{-1} volatile solids was obtained in the subsequent anaerobic digestion, leading to save 501-789 $kJkg^{-1}$ of dry substrate. The feasibility of fungal treatment was also tested for cornstalk delignification in combination with the hydrolysis, through *Trichoderma viride* enzymes and the anaerobic fermentation by *Thermoanaerobacterium thermosaccharolyticum* W16 in order to produce hydrogen via separate saccharification and fermentation (SHF) (Zhao et al. 2012) and simultaneous saccharification and fermentation (SSF) (Zhao et al. 2013). The results revealed the feasibility of the biological delignification showing the highest production of 89.3 mL of hydrogen g^{-1}cornstalk for the SSF in comparison to the 80.3 mL of hydrogen g^{-1} cornstalk obtained through the SHF.

As mentioned above, the solid state fermentation has been also exploited as a process for the production of (hemi)cellulolytic enzymes in combination with following fermentation processes for the production of other upgraded products, such as methane, ethanol and chemicals. Mutschlechner et al. (2015) demonstrated as the optimal combination of inoculation density (2 x 10^8 spores 25 g^{-1} substrate), initial moisture content (70 %) and incubation time (10 days) during the delignification of municipal solid waste promoted a high cellulase activity production by *Trichoderma viride*. This led to an increase of the polysaccharides hydrolysis and nutrients availability, together with a positive effect on the subsequent anaerobic digestion and methane production performed by diluted fermenter sludges. Kiran et al. (2015) exploited the solid state fermentation on waste cake to produce a fungal mash, rich in hydrolytic enzymes, which was used to pretreat food wastes (Table 2). After 24 hours of hydrolysis, the anaerobic digestion of enzymatically pretreated food wastes was carried out, giving a biomethane yield and production rate of ~ 2.3 and 3.5 times higher than that without biological treatment, respectively. They demonstrated as the use of fungal mash in the pretreatment step, directly from solid state fermentation and without any purification, could make more economically competitive the production of biomethane from food waste. Chatzifragkou et al. (2014) tried to develop the biorefinery platform utilizing the by-products from the biodiesel industry, named rapeseed meal (RSM), to produce the 1,3-propanediol (PDO). The fungal strain *Aspergillus oryzae* was inoculated on the RSM at 30 °C in order to promote the (hemi)cellulolityc enzymes production; after 48 hours of fermentation, distilled water was directly added to the solid residues to perform the saccharification and obtain the RSM hydrolysate. The batch cultivation of *Clostridium butyricum* VPI 1718 by using the RSM hydrolysates as fermentation broth gave a 65.5 g $L^{-1}$of PDO with a productivity of 1.15 g $L^{-1}h^{-1}$.

The lignocellulosic wastes treatment through fungal strains for the production of ethanol has been largely exploited (Bhargav et al. 2008; Singhania et al. 2009). In the last decade, bioethanol production has increased rapidly because many countries have issued several “Fuel Ethanol Programs”, aimed at mandating the use of ethanol blends through tax incentives or exemption. In 2014, global bioethanol production churned out a record of 14.3 billion gallons, eclipsing the previous record of 13.9 billion gallons set in 2011 (Srivastava et al. 2015). Mahajen et al. (2014) investigated the solid state fermentation both to produce enzymes and delignify the rice straw for

the ethanol production. Different thermophilic fungal strains were cultivated on rice straw for 7 days at 45 °C and then, the culture extracts were directly used for the subsequent saccharification of the *Myrothecium roridum* treated cornstraw. During the saccharification step, the maximum release of reducing sugars (11.26 mg mL^{-1}) was obtained using the enzymatic cocktail, consisting of cellobiohydrolase, endoglucanase, xylanase, α-arabinofuranosidase, β-xylosidaseand and β-glucosidase, produced by the strain named CM-3T. The resultant hydrolysates were fermented by *Saccharomyces cerevisiae*, reaching the maximum amount of 15.0 g L^{-1} of ethanol.An effective strategy to increase the environmental and economic sustainability of the bioconversion process for ethanol production is represented by the consolidated bioprocessing (CBP), that combines cellulase production, enzymatic hydrolysis and fermentation using an unique microorganism properly engineered to perform all these steps in a single reactor (Zheng et al. 2014). The production of ethanol from lignocellulosic crops by using a fungal strain as unique microorganism was investigated by Okamoto et al. (2011) and Kamei et al. (2012), as reported in Table 2. Okamoto et al. (2011) performed a direct conversion of wheat bran and rice straw into ethanol by using the fungal strain *Trametes hirsuta*. In 6 days, 78.8 % and 57.4 % of the theoretical ethanol yield were achieved on wheat bran and rice straw, respectively. In addition, the white-rot fungus *Phlebia sp.* MG-60 was used as single microorganism for the conversion of oak wood into ethanol (Kamei et al. 2012). Without supplementation of chemicals or enzymes, 40.7% of lignin degradation was reached after 56 days of aerobic incubation, while after 20 days of semi-aerobic saccharification and fermentation, 43.9 % of the maximum theoretical value of ethanol was obtained.

2.2 Bacterial treatment

A single bacterium or a bacterial consortium have been widely investigated for their ability to convert factory wastes in biogas, such as methane and biohydrogen, as widely discussed in the review published by Demirel et al. (2010). Kim and Kim (2013) investigated the production of H_2 and CH_4 from food waste through a novel developed three-stage fermentation process (Table 2). The lactate fermentation effluents (LFE), obtained from the first step of food waste fermentation into lactate, were used as substrate for the growth of *Rhodobacter sphaeroides* KD131 and converted in H_2 by photo-fermentation. Moreover, the remaining residue of LFE were converted in CH_4 through an anaerobic digestion carried out by an anaerobic digester derived from a wastewater treatment plant. The process allowed to convert the 41 % and 37 % of the energy food waste into H_2 and CH_4, respectively. Besides biogas production, recently, the attention was focused on the microbial digestion as pretreatment step for a sustainable conversion of biomass into a spectrum of more competitive bio-based chemicals (Liang et al. 2014; Tang et al. 2015) and biofuels (Zhao C. et al. 2014; Du et al. 2015). In comparison to the fungi, the bacterial strains show lower level of (hemi)cellulolytic activity production, that reduce the loss of cellulose, giving significant advantages during the delignification step (Huang et al. 2013; Brown et al. 2014). Solid state fermentation, carried out by the strain *Bacillus subtilis* NX-2, was performed to covert the agro-industrial waste dry *shiitake* mushroom residues (DSMR) into poly(γ-glutamic acid) (γ-PGA) (Tang et al. 2015) (Table 2). The monosodium glutamate production residues (MGPR) were added to the DSMR as a substitute of glutamate, while the industrial waste glycerol was used in order to increase the carbon source concentration. Under the optimal DSMR-to-

MGPR ratio of 12:8, 115.6 g kg^{-1} γ-PGA was obtained, yield that reached the value of 107.7 g kg^{-1} when the scale-up up to 50 Kg in open air was performed.

Table 2. Added-value products achievable from the upgrading of different wastes.

Wastes	Added-value products	References
Fungal treatment		
Hardwood spent sulphite liquor	Ethanol	Pereira et al. 2012
Sugarcane bagasse	Ethanol	Khuong et al. 2014
Yard trimmings	Methane	Zhao J. et al. 2014
Cornstalk	Hydrogen	Zhao et al. 2012 Zhao et al. 2013
Municipal solid waste	Methane (hemi)cellulolytic anzymes	Mutschlechner et al. 2015
Rice straw	Ethanol (hemi)cellulolytic anzymes	Mahajen et al. 2014
Rapeseed meal	1,3-propanediol (hemi)cellulolytic anzymes	Chatzifragkou et al. 2014
Heat bran Rice straw	Ethanol	Okamoto et al. 2011
Oak wood	Ethanol	Kamei et al. 2012
Bacterial treatment		
Food waste	Methane Hydrogen	Kim and Kim 2013
Agro-industrial	poly(γ-glutamic acid)	Tang et al. 2015
Potato peel waste	Lactic acid Acetic acid Ethanol	Liang et al. 2014
Miscanthus floridulus	Ethanol	Zhao C. et al. 2014
Sweet sorghum stalks	Ethanol	Du et al. 2015
Enzymatic treatment		
Waste cake	Methane	Kiran et al. 2015
Corn stower	Methane	Schroyen et al. 2014
Wheat straw, hemp, flax, corn stover, miscanthus and willow	Methane	Schroyen et al. 2015
Eucalyptus globulus *Pennisetum purpureum*	Ethanol	Gutiérrez et al. 2012
Waste cooking oils	Biodiesel	Talukder et al. 2009
Oil deodorizer distillate	Biodiesel	Su et al. 2014
Residual banana	Glucose, Polyhydroxybutyrate Ethanol	Naranjo et al. 2014
Food wastes	Hydrogen	Han et al. 2015
Paper wastes	Different deinked papers	Lee et al. 2013
Paper wastes	Deinked old newsprint	Virk et al. 2013
Cotton wastes	Scoured cotton fibers	Karapinar and Sariisik, 2004
Cotton wastes	Scoured cotton fibers	Agrawal et al. 2008

Cultures composed of undefined different microrganisms allow to avoid several operative restrictions - such as pH control, sterilization, temperature and nutrients - due to their quick and efficient response to complex conditions, great self-evolution abilities and adaption (Agler et al. 2011). Liang et al. (2014) developed a simple bioconversion of potato peel waste (PPW) into lactic acid, acetic acid and ethanol by using undefined mixed cultures inoculated from wastewater treatment plant sludge (Table 2). Through batch fermentations withouth pH control, 0.22 g g^{-1}, 0.06 g g^{-1}, and 0.05 g g^{-1} of lactic acid, acetic acid and ethanol were obtained.
Mixed microbial culture can be composed of genetically engineered strains or natural consortia. Althogh the engineered strains show high ethanol production and by-products tolerance (Argyros et al. 2011), they exhibit a confined substrate range and more expensive operative conditions in comparison to the natural consortia. Nevertheless, furter investigation are needed to improve the poor ethanol production showed by most of natural consortia (Zuroff et al. 2012). The anaerobic ethanol-producing microbial consortium, named SV79, was isolated from Great Basin hot springs (Nevada, USA) by Zhao C. et al. (2014) and enriched through medium containing the lignocellulosic crop *Miscanthus floridulus* as carbon source. Genera like *Acetivibrio*, *Clostridium*, *Cellulosilyticum*, *Ruminococcus* and *Sporomusa*, belonging to the consortium, were the main responsible for the biomass conversion into ethanol, giving a production of 2.63 mM ethanol g^{-1} of *Miscanthus floridulus* (Table 2). Du et al. (2015) investigated the ethanol production from sweet sorghum stalks of 16 different natural bacterial consortia isolated from several habitats in China (Table 2). Besides to select the HP consortium as the best ethanol producer (2.06 g L^{-1} ethanol), they demonstrated the increment of ethanol production by several microbial consortia when the strain *Pseudoxanthomonas taiwanensis* was added during the fermentation. This strain produced large amount of β-glucosidase enzymes, increasing the cellobiose degradation and reducing the metabolite repression of exoglucanase, thus allowing a better consortium performances. Based on that, they optimized the amount of *Pseudoxanthomonas taiwanensis* in the HP consortium, generating a new consortium named HPP, reaching a maximum ethanol production of 2.5 g L^{-1}.

2.3 Enzymatic treatment

Enzymatic treatments have been adopted for a wide range of biotechnological processes aimed at converting the waste materials into upgraded products. The enzymes represent a green route for the reduction of wastes generation and the energy demand, mandatory for the development of economical and eco-friendly biorefineries.
Several researches have been focused on the enzymes application for lignocellulosic wastes delignification (Kiran et al. 2015; Schroyen et al. 2015; Schroyen et al. 2014; Gutiérrez et al. 2012) as well as for the conversion of oil (Talukder et al. 2009; Su et al. 2014;), food (Naranjo et al. 2014; Han et al. 2015), paper (Lee et al. 2013; Virk et al. 2013) and cotton (Karapinar end Sariisik, 2004; Agrawal et al 2008) wastes into marketable products to be employed in many industrial application, as described below.

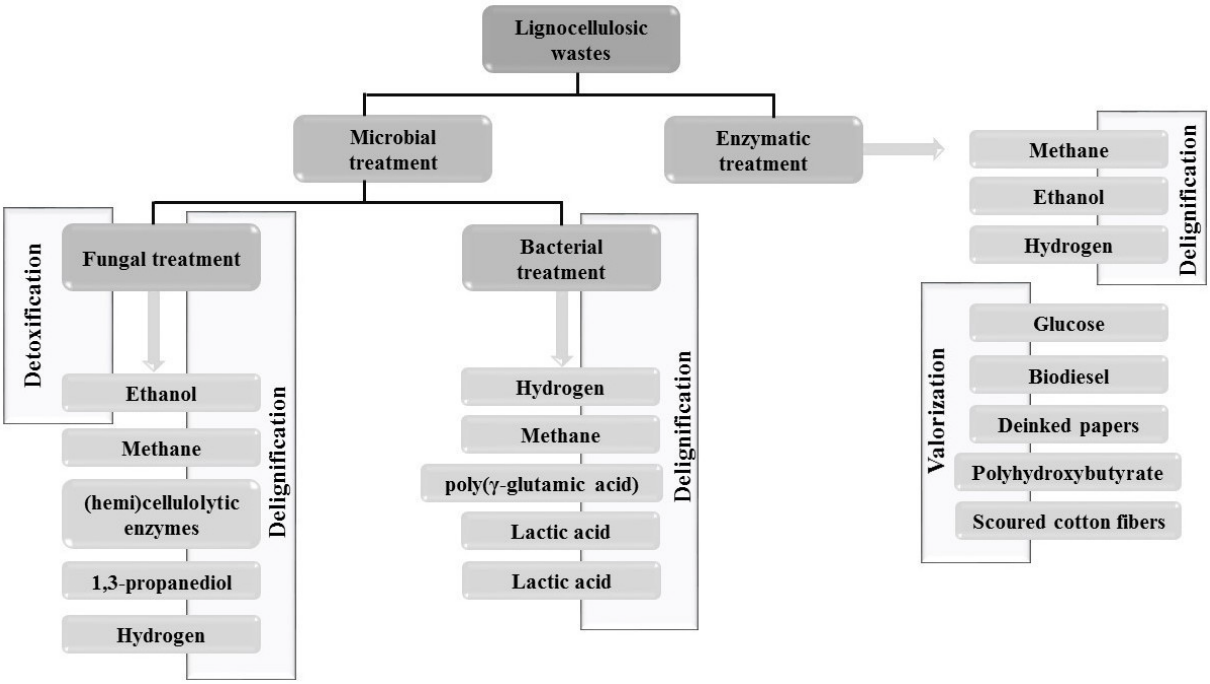

Figure 1. Schematic overview of the biological treatments and the corresponding products described in this review.

2.3.1 Enzymatic delignification

The ligninolytic enzymes can be used for biomass delignification in alternative to fungal and bacterial strains. The application of laccase, lignin peroxidase (LiP), manganese peroxidase (MnP) and versatile peroxidase (VP) (Heinfling et al. 1998; Martínez et al. 2009), not only increases the cellulose accesibility to (hemi)cellulolytic anzymes, but it also allows to recover valuable phenolic compounds derived from lignin degradation (Zakzeski et al. 2009); this combined effect can be exploited to develop an economically and eco-friendly biorefinery system (Roth and Spiess, 2015).

Higher delignification rate and quicker process are two of the main advantages of the use of enzymes in comparison to the microorganisms (Vivekanand et al. 2008). Moreover, the enzymatic delignification can be carried out in wide temperature and pH ranges, the nutrient supplementation is not required and there is no sugars consumption (Ibarra et al. 2006). However, the high cost of the enzymes is the main bottleneck to develop a biorefinery platform in which the bioconversion of lignocellulosic feedstock into added-value bioproducts could be carried out. Many efforts have being focused on the identification of novel lignin-degrading enzymes through advanced metagenomic approaches (Scully et al. 2013), in combination with technologies and tools aimed at enhancing the enzyme performances (Ruiz-Dueñas et al. 2008, Cherry et al. 1999) and reducing the enzymes production costs (Jun et al. 2011). Besides available commercially enzymes, culture supernatant rich in ligninolytic enzymes or mix containing purified enzymes were employed for the biological delignification of lignocellulosic wastes. A cocktail composed of purified laccase derived from *Trametes versicolor* and versatile peroxidase from *Bjerkandera adusta* was tested for the delignification of wheat straw, hemp, flax, corn stover, miscanthus and willow followed by methane production (Schroyen et al. 2015) (Table

2). A strict relation (R^2 = 0.89) between lignin content and biomethane potential (BMP) was demonstrated. They observed a high release of total phenolic compounds (TPC), index of lignin matrix degradation, during the enzymatic treatment of miscanthus and willow, richer in lignin than the other biomasses tested. As a consequence, lower biomethane production (68.8-141.7 NL kg^{-1}) was obtained from miscanthus and willow in comparison to the values (241 - 288 NL kg^{-1}) shown by the other substrates, charachterized by low lignin content. Schroyen et al. (2014) delignified the corn stower with different enzymes, like laccase, manganese peroxidase and versatile peroxidase for different incubation times (0, 6 and 24 h) (Table 2). They observed a biomethane production increament of 25 % after corn stower delignification by using laccase for 24 h, while the peroxidase gave an increment of 17 % after 6 h. Moreover, the treatment was characterized by a low concentration of inhibitors for the subsequent anaerobic digestion.
In most cases, lignin-containing substrates were not oxidized because either they are not able to enter in the activate site of the enzymes or they present a too high redox potential. Based on that, "laccase mediators" were used in combination with laccase to promote the oxidation of non-specific substrates (Hu et al. 2009). Gutiérrez et al. (2012) verified the feasibility of ethanol production from *Eucalyptus globulus* and *Pennisetum purpureum* treated by laccase from *Trametes villosa* in combination with the laccase mediator 1-hydroxybenzotriazole (HBT) (Table 2). The delignification carried out by using 25 U g^{-1} and 2.5% HBT increased the glucose release up to 61 % and 12 % in 72 h and the ethanol production of 4 and 2 g L^{-1} in 17 h for *Eucalyptus* and *Pennisetum,* respectively, in comparison to the no treated ones.

2.3.2 Other wastes upgrading *via enzymatic* conversion.

Oil wastes
Oil wastes are potential feedstock, usable in substitution of the expensive virgin vegetable oils, for the production of biodiesel (BD) through lipase application (Huang et al. 2009). Talukder et al. (2009) developed a conversion of waste cooking oils (WCO) into biodiesel applying a process suitable for a wide range of feedstock showing high free fatty acid (FFA) content, such as waste greases and animal fats (Table 2). In the first step, a lipase from *Candida rugosa* was used to hydrolyze WCO into fatty acid, which was subsequently converted in BD through chemical esterification. A high BD yield, corresponding of 99 %, was reached even if the lipase slightly lost the activity after 5 cycles, determining a decreament of BD yield up to 92 %.
Su et al. (2014) adopted a hydrophobic macroporous resin NKA to immobilize the lipase produced by *Rhizopus oryzae* and improve the enzymes performance during the rapeseed oil deodorizer distillate (RODD) conversion into BD (Table 2). The thermostability and pH-resistance of the immobilized lipase were higher than that of the free ones, giving a BD yield of 98.23 %, value comparable to the yield of 97.46 % obtained by using the commercial enzyme Novozym 435. The system could be efficiently integrated as a part of a RODD biorefinery, since, besides to an efficient BD yield, it reduced by 5 % the loss of vitamin E and sterols, achievable from the FFAs-extracted RODD.

Food wastes
A bioferinery system for the conversion of banana residues (peel and pulp) into glucose, polyhydroxybutyrate (PHB) and ethanol via enzymatic steps was

investigated by Naranjo et al. (2014) (Table 2). The liquefaction of residual banana catalyzed by α-amylase, followed by a saccharification through glucoamylase, allowed the release of glucose, which was subsequently converted into PHB by the strain *Burkholderia sacchari* IPT101 and into bioethanol by *Pichia stipitis*. The results demonstrated the economic feasibility of the process that reduces the PHB, glucose and ethanol production costs to 26 %, 22 % and 53 %, respectively and the global need of energy up to 30.6 %. Moreover, the ethanol yield recovery from residual banana was comparable with that obtained by using corn starch and cane molasses as substrates.

An enzymatic process for the conversion of food wastes from cantinee university into biohydrogen was reported by Han et al. (2015), combining solid state fermentation (SSF) with the dark fermentation (Table 2). SSF of *Aspergillus awamori* and *Aspergillus oryzae* on food waste was exploited to obtain a cocktail of glucoamylase and protease, which were used for the subsequent hydrolysis of fermented food wastes. The obtained hydrolysate, with a final glucose and free amino nitrogen concentration of 36.9 g L^{-1} and 361.3 mg L^{-1}, respectively, was then converted into biohydrogen through dark fermentation by anaerobic sludge collected from a local municipal wastewater treatment plant, reaching 39.14 mL of H_2 g^{-1} of food waste.

Paper wastes

The recycling of paper wastes gives a source of raw fiber that could be reused in many industrial applications within the pulp and paper industrial fields. Among the several processes needed for the paper recycling, the deinking, used to remove ink particles from the paper surface, causes a strong negative impact on the environment due to the large use of chemical agents and the production of damaging emissions (Prasad et al. 1992). The application of enzymes, such as laccases, xylanases, cellulases, esterases, pectinases and amylases is receiving large attention since it involves an improvement of recycled paper properties - like strength and brightness - in combination with a reduction of the environmental impact (Lee et al. 2013; Virk et al. 2013). Lee et al. (2013) evaluated the effects of the enzymatic deinking on different waste papers obtained from the University Sains Malaysia campus. The cellulase A "Amano" 3 and hemicellulase “Amano” 90 (Amano Pharmaceuticals Co. Ltd., Nagoya, Japan) from *Aspergillus niger* were used for the ink removal. Based on the chemical composition of the papers used and the printing techniques employed for the inking, a variable deinking efficiency was observed, giving deinked papers with different mechanical properties. The highest and the lowest deinking efficiency of 86.6 % and 12.9 % were observed for the laser-printed paper and newspaper, respectively. A drainage rate of all kinds of the deinked papers was effectively increased, while unconformable effects were reported for the tensile, burst and tear index. Indeed, the tensile index of magazine paper was improved, while its reduction was observed for the bubble jet-printed paper, photocopy paper and newspaper. A tear index reduction of 21.1 % was observed for the bubble jet-printed whereas a slight increment of 3.1 % was reported for the deinked laser-printed paper. Virk et al. (2013) investigated the feasibility of a methodology that combines enzymatic and physical deinking for the old newsprint. They tested the effectiveness of xylanase from *Bacillus halodurans* FNP 135 and laccase from *Rheinheimera* species, alone and in combination with sonication and microwaving, optimizing the operative conditions such as enzyme dose, pH and treatment time through a surface responce metodology. Combining xylanase and laccase enzymatic activities, the reduction of effective residual ink concentration of

65.8 % was observed, an increment of 37.3 % and 5.8 % in comparison to the use of xylanase or laccase individually. An improvement in optical and strength properties - burst and tear factor, brightness and viscosity - was also observed, coupled with a reduction of 50 % of chemical consumption. The enzymatic treatment combined to a microwave and sonication showed the highest reduction of effective residual ink of 73.9 %, giving a high-quality deinked old newsprint.

Cotton waste

In the textile industry, the scouring treatment was largely employed in order to improve the wettability and the dyeing of the cotton fibers, removing the hydrophobic external cuticula. Usually, hot temperature and chemical agents were exploited for this treatment, requiring large amount of energy and water (Hartzell-Lawson et al. 1998). The application of enzymes for the cotton scouring has received large attention in order to reduce the negative environmental impact of the process (Karapinar and Sariisik, 2004; Agrawal et al. 2008). The wettability and absorbency of cotton fibers after the application of different combinations of pectinase, protease and cellulase were evaluated by Karapinar and Sariisik (2004), taking also the effects on the bleaching and dyeing processes into account. In 60 minutes, a combination of cellulase, protease and pectinase, allowed reaching a wettability comparable with that obtained by applying the mainstream alkaline treatment. Agrawal et al. (2008) developed an efficient scouring strategy at low temperature through a sequential combination of cutinase from *Fusarium solani pisi* and pectinase Bioprep 3000L (Novozymes). The cutinase, acting at 30 °C for 15 min, showed the same wax removal yield obtained with a solvent extraction. Moreover, its enzymatic activity has contrasted the negative increment of hydrophilicity generated by surfactants, like Triton X-100, and increased the pectin removal by improving the pectinase performances as occurs after a n-hexane treatment.

3. Conclusion and future perspectives

Future prospects for the optimization of lignocellulosic wastes bioconversion must involve a more systematic improvement of microorganisms and enzymes performances, with particular attention to the economic feasibility as well as the environmental impact.

Despite its numerous advantages, solid state fermentation is limited by some drawbacks, mainly related to the identification of optimal culture conditions such as substrateconcentration and sterilization, pH and temperature, in addition to the high variability of the process performances (i.e., fungal growth rate and percentage of humidity) and the difficulty for the industrial scale-up. In order to solve the limits in the operating conditions, many efforts are focused on i) discovering new lignocellulolytic microorganisms and/or employing of co-culture systems that are more productive under the stressful conditions, (Agler et al. 2011) and, ii) identifiyng the more suitable paramenters combination through a statistical optimization (Karp et al. 2015). Moreover, improvements on the bioreactors configuration have being carried out to solve engineering problems and make possible the solid-state fermentation in industrial scale (Ali and Zulkali, 2011).

As far as the anzymatic treatment is concerned, the main drawback is related to the high costs of the enzymes and the large amounts required for the industrial applications.

Microorganisms, such as fungi and microbial strains, are exploited as a source of a wide range of enzymatic activities, with the aim to reduce their production costs

through economic processes, such as the solid state fermentation (Bhargav et al. 2008). Nevertheless, naturally produced enzymes show often low performances, like restricted range of pH and temperature, low substrate specificity and biodegradability, all critical features that limit their use and increase the amounts required for accomplish a certain industrial process. Many researches are aimed at producing customized enzyme systems with improved hydrolysis efficiencies. A combination of genomic, transcriptomic and proteomic analysis allows to i) enhance enzymes from natural sources through rational design and directed evolution strategies (Lane and Seelig, 2014), ii) discovering of new enzymes with improved properties by metagenomic approach (Montella et al. 2015); iii) design of artificial enzymes with suitable properties (Liu et al. 2013); and, iv) developing of genetically modified microorganisms more suitable for the production of lignocellulolytic enzymes by optimized expression systems (Lambertz et al. 2014). Recently, the interest is moving towards the expression of in vitro-optimized enzymes in a cell factory; indeed, the use of one microorganism engineered with genes from various sources allows to reduce the impact from an economic point of view (Kondo et al. 2013). However, the real application of genetically modified organisms in the wastes valorization has to face some difficulties related to ethical issues, such as a negative general consumer perception about both the integrity of the manipulated organisms and the effects and the risks caused on the environment and human health after their release. People have a not positive idea of what is considered unnatural, giving more attention to the possible risks than the benefits. This public attitude could be moved towards the acceptance of the engineered organisms through appropriate information and legislation (McHughen and Smythm, 2008; Bawa and Anilakumar, 2013).

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References

1. Agler, M.T., Wrenn, B.A., Zinder, S.H., Angenent, L.T., 2011. Waste to bioproduct conversion with undefined mixed cultures: the carboxylate platform. Trends Biotechnol. 29(2), 70-78.
2. Agrawal, P.B., Nierstrasz, V.A., Bouwhuis, G.H., Warmoeskerken, M.M.C.G., 2008. Cutinase and pectinase in cotton bioscouring: an innovative and fast bioscouring process. Biocatal. Biotransfor. 26(5), 412-421.
3. Ali, H.K.Q., Zulkali, M.M.D., 2011. Design aspects of bioreactors for Solid-state Fermentation: A review. Chem. Biochem. Eng. Q. 25(2), 255-266.
4. Allesch, A., Brunner, P.H., 2014. Assessment methods for solid waste management: A literature review. Waste Manage. Res. 32(6), 461-473.
5. Amore, A., Giacobbe, S., Liguori, R., Faraco, V., 2014. The second generation ethanol production in Rendiconti dell’Accademia Nazionale delle Scienze detta dei XL. Memorie di Scienze Fisiche e Naturali, pp. 113-136.
6. Argyros, D.A., Tripathi, S.A., Barrett, T.F., Rogers, S.R., Feinberg, L.F., Olson, D.G., Foden, J.M., Miller, B.B., Lynd, L.R., Hogsett, D.A., Caiazza, N.C., 2011.

High ethanol titers from cellulose by using metabolically engineered thermophilic, anaerobic microbes. Appl. Environ. Microbiol. 77, 8288-8294.

7. Bawa, A.S., Anilakumar, K.R., (2013). Genetically modified foods: safety, risks and public concerns—a review. J. Food Sci. Technol. 50(6), 1035-1046.
8. Bhargav, S., Panda, B.P., Ali, M., Javed S., 2008. Solid-state Fermentation: An Overview. Chem. Biochem. Eng. Q. 22(1), 49-70.
9. Brown, M.E., Chang, M.C.Y., 2014. Exploring bacterial lignin degradation. Curr. Opin. Chem. Biol. 19, 1-7.
10. Chandel, A.K., Gonçalves, B.C.M., Strap, J.L., da Silva S.S., 2015. Biodelignification of lignocellulose substrates: An intrinsic and sustainable pretreatment strategy for clean energy production. Crit. Rev. Biotechnol. 35(3), 281-93.
11. Chatzifragkou, A., Papanikolaou, S., Kopsahelis, N., Kachrimanidou, V., Dorado, M.P., Koutinas, A.A., 2014. Biorefinery development through utilization of biodiesel industry by-products as sole fermentation feedstock for 1,3-propanediol production. Bioresour. Technol. 159, 167-175.
12. Cherry, J.R., Lamsa, M.H., Schneider, P., Vind, J., Svendsen, A., Jones, A., Pedersen, A.H., 1999. Directed evolution of a fungal peroxidase. Nat. Biotechnol. 17, 379-384.
13. Demirel, B., Scherer, P., Yenigun, O., Onay T.T., 2010. Production of methane and hydrogen from biomass through conventional and high-rate anaerobic digestion processes. Crit. Rev. Env. Sci. Technol. 40(2), 116-146.
14. Du, R., Yan, J., Li, S., Zhang, L., Zhang, S., Li, J., Zhao, G., Qi P., 2015. Cellulosic ethanol production by natural bacterial consortia is enhanced by *Pseudoxanthomonas taiwanensis*. Biotechnol. Biofuels 8, 10-19.
15. FitzPatrick, M., Champagne, P., Cunningham, M.F., Whitney, R.A., 2010. A biorefinery processing perspective: Treatment of lignocellulosic materials for the production of value-added products. Biores. Technol. 101, 8915-8922.
16. Gavrilescu, M., 2014. Biorefinery systems: An overview. Bioenergy Research*:* Advances and Applications, chapter 14, pp. 219-241.
17. Gujarathia, A.M., Sadaphal, A., Bathec, G.A., 2015. Multi-objective optimization of solid state fermentation process. Mater. Manuf. Process. 30 (4), 511-519.
18. Gutiérrez, A., Rencoret, J., Cadena, E.M., Rico, A., Barth, D., del Río, J.C., Martínez, Á.T., 2012. Demonstration of laccase-based removal of lignin from wood and non-wood plant feedstocks. Bioresour. Technol. 119, 114-122.
19. Han, W., Ye, M., Zhu, A.J., Zhao, H.T., Li, Y.F., 2015. Batch dark fermentation from enzymatic hydrolyzed food waste for hydrogen production. Bioresour. Technol. 191, 24-29.
20. Hartzell-Lawson, M.M., Hsieh, Y.L., 1998. Enzymatic scouring to improve cotton fabric wettability. Text. Res. J. 68(4), 233-241.
21. Heinfling, A., Ruiz-Duefias, F.J., Martinez, M.J., Bergbauer, M., Szewzyk, U., Martinez, A.T., 1998. A study on reducing substrates of manganese-oxidizing peroxidases from *Pleurotus eryngii* and *Bjerkundera adusta*. FEBS Len. 428, 141-146.
22. Hu, M.R., Chao, Y.P., Zhang, G.Q., Xue, Z.Q., Qianù S., 2009. Laccase-mediator system in the decolorization of diVerent types of recalcitrant dyes. J. Ind. Microbiol. Biotechnol. 36, 45-51.
23. Huang, C., Zong, M.H., Wu, H., Liu Q.P., 2009. Microbial oil production from rice straw hydrolysate by *Trichosporon fermentans*. Bioresour. Technol. 100(19), 4535-4538.

24. Huang, X.F., Santhanam, N., Badri, D.V., Hunter, W.J., Manter, D.K., Decker, S.R., Vivanco, J.M., Reardon, K.F., 2013. Isolation and characterization of lignin-degrading bacteria from rainforest soils. Biotechnol. Bioeng. 110(6), 1616-1626.
25. Ibarra, D., Romero, J., Martínez, M.J., Martínez, A.T., Camarero, S., 2006. Exploring the enzymatic parameters for optimal delignification of eucalypt pulp by laccase mediator. Enzyme Microb. Technol. 39, 1319-27.
26. Jacquet, N., Haubruge, E., Richel, A., 2015. Production of biofuels and biomolecules in the framework of circular economy: A regional case study. Waste Manage. Res. 33(12), 1121-1126.
27. Jun, H., Kieselbach, T., Jönsson, L.J., 2011. Enzyme production by filamentous fungi: analysis of the secretome of *Trichoderma reesei* grown on unconventional carbon source. Microb. Cell. Fact. 10, 68-78.
28. Kamei, I., Hirota, Y., Meguro S., 2012. Integrated delignification and simultaneous saccharification and fermentation of hard wood by a white-rot fungus, *Phlebia sp.* MG-60. Bioresour. Technol. 126, 137-141.
29. Karapinar, E., Sariisik, M.O., 2004. Scouring of cotton with cellulases, pectinases and proteases. Fibres. Text. East. Eur. 12(3), 79-82.
30. Karp, S.G., Faraco, V., Amore, A., Letti, L.A.J., Soccol, V.T., Soccol, C.R., 2015. Statistical optimization of laccase production and delignification of sugarcane bagasse by *Pleurotus ostreatus* in Solid-State Fermentation. BioMed Research International, Volume 2015, Article ID 181204, 8 pages http://dx.doi.org/10.1155/2015/181204.
31. Khuong, L.D., Kondo, R., De Leon, R., Anh, T.K., Meguro, S., Shimizu, K., Kamei, I., 2014. Effect of chemical factors on integrated fungal fermentation of sugarcane bagasse for ethanol production by a white-rot fungus, *Phlebia* sp. MG-60. Bioresour. Technol. 167, 33-40.
32. Kim, D.H., Kim M.S., 2013. Development of a novel three-stage fermentation system converting food waste to hydrogen and methane. Bioresour. Technol. 127, 267-274.
33. Kiran, E.U., Trzcinski, A.P., Liu, Y., 2015. Enhancing the hydrolysis and methane production potential of mixed food waste by an effective enzymatic pretreatment. Bioresour. Technol. 183, 47-52.
34. Kondo, A., Ishii, J., Harac, K.Y., Hasunuma, T., Matsuda, F., 2013. Development of microbial cell factories for bio-refinery through synthetic bioengineering. J. Biotechnol. 163, 204-216.
35. Lambertz, C., Garvey, M., Klinger, J., Heesel, D., Klose, H., Fischer, R., Commandeur U., 2014. Challenges and advances in the heterologous expression of cellulolytic enzymes: a review. Biotechnol. Biofuels 7, 135-149.
36. Lane, M.D., Seelig, B., 2014. Advances in the directed evolution of proteins. Curr. Opin. Chem. Biol. 22, 129-136.
37. Lee, C.K., Ibrahim, D., Omar, I.C., 2013. Enzymatic deinking of various types of waste paper: Efficiency and characteristics. Process Biochem. 48, 299-305.
38. Liang, S., McDonald, A.G., Coats E.R., 2014. Lactic acid production with undefined mixed culture fermentation of potato peel waste. Waste Manage. 34, 2022-2027.
39. Liguori, R., Amore, A., Faraco, V., 2013. Waste valorization by biotechnological conversion into added value products. Appl. Microbiol. Biotechnol. 97(14), 6129–6147.

40. Liu, G., Qin, Y., Li, Z., Qu, Y., 2013. Development of highly efficient, low-cost lignocellulolytic enzyme systems in the post-genomic era. Biotechnol. Adv. 31, 962-975.
41. Mahajan, C., Chadha, B.S., Nain, L., Kaur A., 2014. Evaluation of glycosyl hydrolases from thermophilic fungi for their potential in bioconversion of alkali and biologically treated *Parthenium hysterophorus* weed and rices traw into ethanol. Bioresour. Technol. 163, 300-307.
42. Martínez, Á.T., Ruiz-Dueñas, F.J., Martínez, M.J., del Río, J.C., Gutiérrez A., 2009. Enzymatic delignification of plant cell wall: from nature to mill. Curr. Opin. Biotech. 20, 348-357.
43. McHughen, A., Smythm, S., 2008. US regulatory system for genetically modified [genetically modified organism (GMO), rDNA or transgenic] crop cultivars. Plant Biotech. J. 6, 2-12.
44. Melikoglua, M., Lina, C.S.K., Webb, C., 2013. Stepwise optimisation of enzyme production in solid state fermentation of waste bread pieces. Food. Bioprod. Process. 91, 638-646
45. Montella, S., Amore, A., Faraco, V., 2015. Metagenomics for the development of new biocatalysts to advance lignocellulose saccharification for bioeconomic development. Crit. Rev. Biotechnol. Published online 16 September 2015. DOI: 10.3109/07388551.2015.1083939.
46. Moreno, A.D., Ibarra, D., Alvira, P., Tomás-Pejó, E., Ballesteros, M., 2015. A review of biological delignification and detoxification methods for lignocellulosic bioethanol production. Crit. Rev. Biotechnol. 35(3), 342-354.
47. Mutschlechner, M., Illmer, P., Wagner A.O., 2015. Biological pre-treatment: Enhancing biogas production using the highly cellulolytic fungus *Trichoderma viride*. Waste Manage. 43, 98-107.
48. Naranjo, J.M., Cardona, C.A., Higuita J.C., 2014. Use of residual banana for polyhydroxybutyrate (PHB) production: Case of study in an integrated biorefinery. Waste Manage. 34, 2634-2640.
49. Okamoto, K., Nitta, Y., Maekawa, N., Yanase H., 2011. Direct ethanol production from starch, wheat bran and rice straw by the white rot fungus *Trametes hirsuta*. Enzyme Microb. Tech. 48, 273-277.
50. Pereira, S.R., Ivanuša, Š., Evtuguin, D.V., Serafim, L.S., Xavier, A.M.R.B., 2012. Biological treatment of eucalypts pent sulphite liquors: A way to boost the production of second generation bioethanol. Bioresour. Technol. 103, 131-135.
51. Prasad, D.Y., Heitmann, J.A., Joyce, T.W., 1992. Enzyme deinking of black and white letterpress printed newsprint waste. Prog. Pap. Recycl. 1(3), 21-30.
52. Renewables 2015 Global Status Report (Paris: REN21 Secretariat). ISBN 978-3-9815934-6-4.
53. Roth, S., Spiess, A.C., 2015. Laccases for biorefinery applications: a critical review on challenges and perspectives. Bioprocess Biosyst. Eng. 38, 2285-2313.
54. Ruiz-Dueñas, F.J., Morales, M., Mate, M.J., Romero, A., Martínez, M.J., Smith, A.T., Martínez, A.T., 2008. Site-directed mutagenesis of the catalytic tryptophan environment in *Pleurotus eryngii* versatile peroxidase. Biochemistry 47, 1685-1695.
55. Salvachúa, D., Prieto, A., López-Abelairas, M.a., Lu-Chau, T., Martínez, A.T., Martínez, M.a.J.s., 2011. Fungal pretreatment: an alternative in second-generation ethanol from wheatstraw. Bioresour. Technol. 102, 7500-7506.

56. Schroyen, M., Vervaeren, H., Van Hulle, S.W.H., Raes, K., 2014. Impact of enzymatic pretreatment on corn stover degradation and biogas production. Bioresour. Technol. 173, 59-66.
57. Schroyen, M., Vervaeren, H., Vandepitte, H., Van Hulle, S.W.H., Raes, K., 2015. Effect of enzymatic pretreatment of various lignocellulosic substrates on production of phenolic compounds and biomethane potential. Bioresour. Technol. 192, 696-702.
58. Scully, E.D., Geib, S.M., Hoover, K., Tien, M., Tringe, S.G., Barry, K.W., del Rio, T.G., Chovatia, M., Herr, J.R., Carlson, J.E., 2013. Metagenomic profiling reveals lignocellulose degrading system in a microbial community associated with a wood-feeding beetle. PlosOne 8(9) e73827- e73849.
59. Singhania, R.R., Patel, A.K., Soccol, C.R., Pandey, A., 2009. Recent advances in solid-state fermentation. Biochem. Eng. J. 44(1), 13-18.
60. Srivastava, N., Rawat, R., Oberoi, H.S., Ramteke, P.W., 2015. A review on fuel ethanol production from lignocellulosic biomass. Int. J. Green Energy 12(9), 949-960.
61. Su, F., Li, G., Zhang, H., Yan, Y., 2014. Enhanced performance of *Rhizopus oryzae* lipase immobilized on hydrophobic carriers and its application in biorefinery of rapeseed oil deodorizer distillate. Bioenerg. Res. 7, 935-945.
62. Talukder, Md.M.R., Wu, J.C., Chua L.P.L., 2010. Conversion of waste cooking oil to biodiesel via enzymatic hydrolysis followed by chemical esterification. Energy Fuels 24, 2016-2019.
63. Tang, B., Xu, H., Xu, Z., Xu, C., Xu, Z., Lei, P., Qiu, Y., Liang, J., Feng, X., 2015. Conversion of agroindustrial residues for high poly(γ-glutamic acid) production by *Bacillus subtilis* NX-2 via solid-state fermentation. Bioresour. Technol. 181, 351-4.
64. United Nations Environment Programme (UNEP) and International Solid Waste Association (ISWA) (2015). Global Waste Management Outlook. Wilson DC (Ed) Authors: Wilson DC, Rodic L, Modak P, Soos R, Carpintero A, Velis CA, Iyer M and Simonett O. UNEP International Environment Technology Centre: Osaka, September 2015.
65. Velis, C.A., 2015. Circular economy and global secondary material supply chains. Waste Manage. Res. 33(5), 389-391.
66. Virk, A.P., Puri, M., Gupta, V., Capalash, N., Sharma, P., 2013. Combined enzymatic and physical deinking methodology for efficient eco-friendly recycling of old newsprint. PLoSOne 8(8), e72346-e72354.
67. Vivekanand, V., Dwivedi, P., Sharma, A., Sabharwal, N., Singh, R.P., 2008. Enhanced delignification of mixed wood pulp by *Aspergillus fumigatus* laccase mediator system. World J. Microbiol. Biotechnol. 24, 2799-804.
68. Wilson, D.C., Velis, C.A., 2015. Waste management – still a global challenge in the 21st century: An evidence-based call for action. Waste Manage. Res. 33(12), 1049-1051.
69. Zakzeski, J., Bruijnincx, P.C., Jongerius, A.L., Weckhuysen, B.M., 2009. The catalytic valorization of lignin for the production of renewable chemicals. Chem. Rev. 110, 3552-3599.
70. Zhao, C., Deng, Y., Wang, X., Li, Q., Huang, Y., Liu, B., 2014. Identification and characterization of an anaerobic ethanol-producing cellulolytic bacterial consortium from great basin hot springs with agricultural residues and energy crops. J. Microbiol. Biotechnol. 24(9), 1280-1290.

71. Zhao, J., Ge, X., Vasco-Correa, J., Li, Y., 2014. Fungal pretreatment of unsterilized yard trimmings for enhanced methane production by solid-state anaerobic digestion. Bioresour. Technol. 158, 248-252.
72. Zhao, L., Cao, G.L., Wang, A.J., Guo, W.Q., Ren, H.Y., Ren, N.Q., 2013. Simultaneous saccharification and fermentation of fungal pretreated corn stalk for hydrogen production using *Thermoanaerobacterium thermosaccharolyticum* W16. Bioresour. Technol 145, 103-107.
73. Zhao, L., Cao, G.L., Wanga, A.J., Ren, H.Y., Dong, D., Liu, Z.N., Guan, X.Y., Xu, C.J., Ren N.Q., 2012. Fungal pretreatment of corn stalk with *Phanerochaete chrysosporium* for enhancing enzymatic saccharification and hydrogen production. Bioresour. Technol. 114, 365-369.
74. Zheng, Y., Zhao, J., Xu, F., Li Y., 2014. Pretreatment of lignocellulosic biomass for enhanced biogas production. Prog. Energ. Combust. 42, 35-53.
75. Zuroff, T.R., Curtis, W.R., 2012. Developing symbiotic consortia for lignocellulosic biofuel production. Appl. Microbiol. Biotechnol. 93, 1423-1435.

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Research paper

Transcriptome analysis of *Sulfolobus solfataricus* infected with two related fuselloviruses reveals novel insights into the regulation of CRISPR-Cas system

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ABSTRACT

Fuselloviruses SSV1 and SSV2 are model systems to investigate virus–host relationships in stably infected cells thanks to their temperate nature. Although they are very similar in morphology, genome organization and gene synteny, their replication is induced by different stimuli, i.e.: by UV-light exposure (for SSV1) and by the growth progression of the host (for SSV2). In this study, we have analysed global gene expression in SSV1- and SSV2-lysogens of *Sulfolobus solfataricus* P2 in the absence of any stimuli. Additionally, the interplay among SSV1, SSV2 and the host has been investigated in a double-infected strain to explore both virus–host and virus–virus interactions. Whereas SSV1 did not induce major changes of the host gene expression, SSV2 elicited a strong host response, which includes the transcriptional activation of CRISPR loci and *cas* genes. As a consequence, a significant decrease of the SSV2 copy number has been observed, which in turn led to provirus-capture into the host chromosome. Results of this study have revealed novel aspects of the host–viral interaction in the frame of the CRISPR-response.

1. Introduction

The majority of organisms is susceptible to viral infection and accordingly it has been predicted that viruses inhabit several niches worldwide [1,2]. This makes viruses, especially those infecting prokaryotes (i.e. *Archaea* and *Bacteria*), the most predominant biological entity on Earth [3]. Soon after the discovery of the archaeal domain, many viruses and virus-like particles have been isolated from extremely hot, low pH or hypersaline niches [4,5]. Intriguingly, these viruses exhibit unique morphologies compared to those of bacteriophages, and fall into ten new virus families in the current classification [4,6,7]. For instance, spindle-shaped viruses are exceptional on their own since this morphotype is a hallmark of viruses infecting archaeal microorganisms [8] and it has never been described for bacteriophages or eukaryal viruses.

Spindle-shaped viruses infecting organisms of the genus *Sulfolobus* (SSVs) belong to the family *Fuselloviridae*, which comprises so far ten members (SSV1, SSV2, SSV3, SSV4, SSV5, SSV6, SSV7, SSV8, SSV9 and ASV1) [5,9]. *Sulfolobus* spindle-shaped virus 1 (SSV1) is a model virus for investigating virus–host relationship, since it is the only UV-inducible archaeal virus isolated to date [9–11]. Analysis of SSV1 transcription has laid the basis for understanding how archaeal gene expression is regulated [12,13]. Moreover, its genome has served as a backbone for the construction of vectors for genetic manipulation and gene expression in *Sulfolobus* [9]. SSV1 is a temperate virus that, upon infection, establishes a stable coexistence with the host by keeping its copy number low and constant throughout the growth of infected cells. It is worth noting that, unlike lambda-lysogens, in which only proviruses exist, SSV1-lysogens carry both a provirus (an integrated viral genome in the host chromosome) and a few episomal copies of the viral DNA [14,15]. Consequently, viral progeny is

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constitutively produced at low level even in the absence of the inducing stimulus (i.e. UV-light exposure). Therefore, the lysogenic state of SSV1 is better defined as a carrier state [11,16]. The transcriptional map of SSV1 highlighted the chronological expression of viral genes as consequence of the UV exposure [10] and helped speculating about the function of their products. However, so far, the general lack of sequence similarity with other proteins in public databases has hindered functional studies of these viral genes. In fact, only a limited number of proteins encoded by SSV1 or other fuselloviruses have been functionally and/or structurally characterized [11,17–24].

On the other hand, although SSV2 resembles SSV1 in the shape of the viral particle as well as in genome organization and gene synteny, its replication is not induced by an external stimulus as for SSV1. Rather, the SSV2 copy number is kept constant and low (around 1–3 copies per cell) until the native host *Sulfolobus islandicus* REY15/4 enters into the stationary growth phase, when a steep increase of the copy number occurs (25–50 copies per cell) [25]. This effect has not been observed for the permissive host *Sulfolobus solfataricus*, in which SSV2 shows a copy number comparable to that of the induced state of the natural host. This led to the hypothesis that a transcription factor encoded by the natural host *S. islandicus* was involved in the regulation of the viral replication induction and this factor is presumably absent in the foreign host *S. solfataricus* [25].

Recently, a transcriptome analysis has been carried out to define the gene expression pattern of SSV2 in the early stages of infection of *S. solfataricus* cells (up to 9 h post infection) [26]. This allowed detecting seven non-overlapping transcripts that were termed after the SSV1 counterparts. Although, genome transcription occurred (as for SSV1) in a temporal fashion, early genes were not adjacently located and displayed a distributive pattern of expression [26]. Since the study by Ren et al., is limited to a short post-infection time window, genes responsible for the maintenance of the SSV2 carrier state have not been identified.

Only a few global gene expression analyses of hosts infected with archaeal viruses have been reported [10,26–29]. Worthmentioning are those performed on the lytic *Sulfolobus islandicus* rod-shaped virus 2 (SIRV2), which exhibits a temporal pattern of gene expression [28] as well as on the *Sulfolobus* turreted icosahedral virus (STIV), which does not show any temporal regulation [27]. Viral–host interactions in *Crenarchaea* have been successfully investigated at gene expression level both for lytic and temperate viruses, using as host *S. solfataricus* P2. Since it has been shown that several *S. solfataricus* strains isolated in Italy are susceptible to infection by all fuselloviruses [30], this host is a suitable crenarchaeal model to study fusellovirus–host relationships. Moreover, the strain P2 harbours the CRISPR-Cas antiviral defense system, which is one of the main players of the virus–host arms race. In brief, to achieve viral immunity, DNA sequences of invading genetic elements (i.e. protospacers) are integrated in the array of a CRISPR locus in a process known as adaptation. Transcription of these loci produces crRNAs that form ribonucleoprotein complexes with Cas proteins. Foreign nucleic acids are detected and degraded by these complexes in the interference stage [31].

The genome of *S. solfataricus* P2 harbours six CRISPR loci (from A to F) and *cas* gene cassettes encoding for up to seven different interference complexes, i.e. of subtypes I-A (Cascade complex), III-B (CMR complex) and III-A [31]. Whereas ribonucleoprotein complexes of subtypes IA and III-A exert DNA recognition and degradation, those belonging to the subtype III-B cleave RNA in a sequence-specific manner. These features make *S. solfataricus* P2 an exceptional model to investigate the modulation of the CRISPR-Cas system activity in response to the infection by different viruses. Herein we report a whole-transcriptome analysis that highlights gene expression remodelling in *S. solfataricus* upon the establishment of SSV1 and SSV2 into the host cells. Previous studies have been carried out to investigate on variation of gene expression in the immediate aftermath of SSV2 infection (up to 9 h later) [26] or upon UV-stimulus in SSV1 lysogens [10]. We show that once SSV1 and SSV2 established a carrier state, the host response elicited by the two viruses is very different. Noteworthy, the up-regulation of CRISPR-Cas system occurs only in cells infected with SSV2.

2. Materials and methods

2.1. Strains, media and growth conditions

SSV1-, SSV2- and SSV1/SSV2-infected strains of *S. solfataricus* P2 were generated, as described elsewhere [11,25], using as host the uracil auxotrophic mutant InF1 [32]. Cultures were grown aerobically in TYSU, i.e. a glycine-buffered Brock's basal salt solution supplemented with 0.1% tryptone, 0.05% yeast extract, 0.2% sucrose and 0.002% uracil (w/v); the pH was adjusted to 3.2 with H_2SO_4 [32]. Incubation was conducted in 250-ml Erlenmeyer flasks at 75 °C, with a shaking rate of 150 rpm in an Innova 3100 Water bath shaker (New Brunswick Scientific Corp).

Aliquots from frozen cultures of the uninfected InF1 and the infected strains (SSV1-InF1, SSV2-InF1 and SSV1/SSV2-InF1) were revitalized by inoculating in TYSU medium. Cell growth was spectrophotometrically monitored at 600 nm (OD_{600}) throughout the cultivation by means of a Variant Cary® 50 Bio UV/Visible Spectrophotometer (McKinley Scientific). Once reached 0.4 OD_{600}, cultures were diluted to 0.05 OD_{600}, incubated back to 75 °C and samples collected at 0.4 OD_{600} (early exponential phase) and 1.2 OD_{600} (late exponential phase). Cellular pellets were obtained by centrifugation at 3000 × *g* for 10 min using the Centrifuge 5810R (Eppendorf) and treated for total DNA and RNA preparations.

To isolate single clones from the SSV2-InF1 strain, serial dilutions of this culture were plated on TYSU-Gelrite and incubated at 75 °C. Isolated colonies appeared on the plate surface after 7–10 days (about 100 colonies per plate). Several colonies were inoculated in liquid medium (about 10 per plate), let to grow until 0.4 OD_{600} and culture supernatant tested by plaque assay for viral titre determination, using the uninfected InF1 strain as lawn and the supernatant of the SSV2-InF1 culture as a control. Single clones showing a lower viral titre were streaked on plates three times in the attempt to isolate SSV2-cured cells.

2.2. RNA extraction, cDNA synthesis and labelling

Total RNA samples were prepared using the TRIzol reagent (Sigma Aldrich®) and carried-over DNA was digested using Turbo™ DNase (Ambion®), according to the manufacturer's instructions. The enzyme was thermal inactivated at 70 °C for 10 min after the addition of 5 mM EDTA (Ethylene Diamine Tetraacetic Acid). DNA-free RNA samples were purified by phenolic extraction and ethanol precipitation. RNA pellets were dissolved in nuclease-free water and both concentration and integrity were checked through: i) electrophoresis on denaturing, formaldehyde-containing 2.0% agarose gel and ii) determination of the 260nm/280 nm adsorption ratio using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). Only samples showing ratios between 2.1 and 1.9 were used for cDNA synthesis.

cDNA labelled with 5-(3-aminoallyl)-dUTP (aa-dUTP) was generated using the Amersham CyScribe Post Labeling Kit (GE Healthcare) according to the manufacturer's directions, with few modifications as follows: 4 µl of random hexamers primers, 1 µl

random nonamers primers, 10–15 μg total RNA and nuclease-free water were mixed in a final volume of 11 μl. The mixture was denatured at 70 °C for 5 min and chilled at room temperature for 10 min. Four microlitres of 5 × CyScribe buffer, 1 μl dNTP mix, 1 μl aa-dUTP, 2 μl DTT 0.1 M and 1 μl CyScribe reverse transcriptase, were added to the reaction mixture and incubated at 42 °C for 90 min. Afterwards, the RNA template was degraded by adding 2 μl of 2.5 M NaOH and incubating at 37 °C for 15 min. The allyl-dUTP cDNA sample was purified using the Illustra Cyscribe GFX Purification kit (GE Healthcare) after neutralizing the reaction mixture with the addition of 10 μl of 2 M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid). The concentration of the purified cDNA was spectrophotometrically determined.

The CyDye labelling of the amino allyl-modified cDNA was achieved using the Amersham CyScribe Post Labeling Kit (GE Healthcare) and purification of the labelled cDNA was carried out using the MinElute® PCR purification kit (Qiagen), following the manufacturer's instructions. Reference (ref) and experimental (exp) cDNA samples were labelled with cyanine-3 (Cy-3) and cyanine-5 (Cy-5), respectively. The ratio between the CyDye-labelled and the total cDNAs was used to monitor the labelling efficiency. Only reference and experimental cDNA samples showing similar labelling efficiency were co-hybridized on the same slide. For each hybridization reference and experimental samples are indicated in the text (Section 3, Results and discussion).

2.3. Microarray hybridization and data analysis

Customized microarray slides harbouring about 7000 spots were designed by the *Sulfolobus* genome chips consortium and manufactured by Ocimum Biosolutions (Hyderabad). A single array includes probes, spotted in duplicate, for 3042 *S. solfataricus* P2 genes, for several crenarchaeal viruses, three plasmids as well as for human and *Arabidopsis thaliana* sequences as negative controls [26–28]. The microarray slide was first dipped into a coupling jar containing 25 ml of prewarmed prehybridization solution (1% BSA; 5 × SSC; 0.1% SDS) and then incubated at 42 °C for 40 min under shaking (Thermo-Electron Corporation). The prehybridized slide was washed three times with distilled water and once with 100% isopropanol. Finally, the slide was dried by centrifugation and a LifterSlip coverslip was applied onto the array-area.

The hybridization solution, i.e. 60% deionized formamide, 7 × SSC (1.0 M NaCl, 0.1 M sodium citrate), 2% SDS, 0.2 μg/μl herring sperm DNA and 0.2 μg/μl tRNA was incubated at 95 °C for 2 min and chilled on ice for 1 min, before adding equal amounts of reference and experimental cDNAs (50 pmol CyDye for each). Subsequently, the hybridization mixture was carefully injected between the array surface and the coverslip. The slide was sealed in a hybridization chamber and incubated at 42 °C for 18 h. Washing of the hybridized slide was performed with: i) a prewarmed solution A (2 × SSC; 0.1% SDS), at 42 °C for 5 min under gentle shaking; ii) a prewarmed solution B (0.1 × SSC; 0.1% SDS) for 20 min at 42 °C and iii) a solution C (0.1 × SSC) for five times at room temperature. Finally, the slide was dried by centrifugation and immediately scanned using the Array Wor$^{s}_{x}$ (Applied Precision). Data analysis was conducted by ImaGene® v. 9.0 (BioDiscovery) using default settings, and included the following steps: data import, background adjustment, normalization, summarization and quality assessment.

Up to four independent experiments were carried out for each reference/experimental cDNA couple. Moreover, since each probe is present in duplicate on a slide, the fold-change variation for each gene is the average among the collected data points. In particular, the software Imagene® gives a $\log_2$ ratio value for each analysed spot:

$$[\log_2(\text{FCy-5 exp})] - [\log_2(\text{Fcy-3 ref})] = \log_2(\text{Fcy5 exp/Fcy3 ref})$$

where "F" indicates the normalized fluorescence intensity of the Cy-3 and Cy-5 detected in a given spot. The variation of the gene expression fold (VGEF) is calculated through the following equation:

$$\text{VGEF} = 2^{\log 2(\text{Fcy-5 exp/Fcy-3 ref})}$$

Only genes with a VGEF of $\geq$2 as well as a p value of <0.05 were regarded as differentially expressed.

2.4. PCR analysis of CRISPR loci leader-proximal regions and of the SSV2 integration site

A fundamental step in the CRISPR-Cas system is the adaptation, which consists of the Cas proteins-mediate incorporation of new spacers that occurs immediately downstream the leader sequence of a given CRISPR locus. With the purpose of detecting new spacers integration at the leader-proximal regions of all CRISPR loci (A–F) in the *S. solfataricus* genome, six primer couples were used (Table S1). In particular, these oligonucleotides were designed to amplify the first 5–8 repeat-spacer units located immediately downstream the leader sequence [33]. Thus, integration of new spacers will produce longer PCR products than the control sample (uninfected InF1).

Total DNA samples were prepared using the DNeasy tissue kit (Qiagen), following the manufacturer's instructions and their concentration was spectrophotometrically measured using a Nanodrop 2000 Spectrophotometer (Thermo-Scientific). PCR master mixes were prepared as follows: 1 × Taq Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.6 μM *primer-fw*, 0.6 μM *primer-rv* and 0.05 U/μl of Taq DNA Polymerase (Thermo Scientific). The thermal cycling protocol was as follows: an initial denaturation step of 5 min at 95 °C, followed by 35 cycles of 1 min at 95 °C, 1 min at 50 °C, and 1 min at 72 °C. A final step at 72 °C has been carried out for 10 min at the end of the 35th cycle. A negative control (no template) was also included in the analysis. PCR products were analysed in a 1% agarose gel.

In parallel, to check the occupancy of the SSV2-integration site in the *S. solfataricus* genome, a PCR analysis was carried out using the primers SSV2attApO1, SSV2attApO2 and SSV2attApO3 [25], whose sequences are listed in Table S1. The reactions were set up (as described above) except the annealing temperature was of 55 °C.

2.5. Semi-quantitative PCR analysis of the SSV2 DNA content

Cell pellets of the initial population InF1-SSV2 and of the isolated clones showing a lower SSV2 titre (clone 1, 1Q and $1Q_1$) were treated for total DNA extraction using the DNeasy tissue kit (Qiagen). To follow the variation of the SSV2 DNA content among the different clones, a semi-quantitative PCR analysis was performed. Two primer couples were designed using Primer3 software (available at the website: http://bioinfo.ut.ee/primer3-0.4.0/), in order to amplify: (i) a 108-bp region of the host single-copy gene *orc1* and (ii) a 238-bp region of the SSV2 single-copy gene *vp3* (Table S1). A master mix was prepared and PCRs were carried out as described elsewhere [34], using the following cycling protocol: an initial denaturation step of 5 min at 95 °C, followed by 30 cycles of 40 s at 95 °C, 40 s at 62 °C, and 1 min at 72 °C. For each reaction, tubes were taken from the thermocycler at the 20th, 25th and 30th cycle of amplification. PCR products were analysed by agarose gel

electrophoresis in 1 × TAE buffer pH 8.0 (40 mM Tris, 20 mM acetic acid and 1 mM EDTA).

3. Results and discussion

3.1. Viral gene expression in the infected strains

Previous studies were carried out in the immediate aftermath of the viral infection [26], on lytic viruses [27–29] or upon induction of the viral replication [10], and very little is known about the viral/host gene expression in the carrier state. Two fuselloviruses, SSV1 and SSV2, were chosen as model in this study since they are not lytic and are therefore suitable for transcriptomic analysis in stably infected populations. Furthermore, the double-infected SSV1/SSV2-InF1 strain is a ternary system, which is suitable for analysing the mutual effect of the two viruses on each other as well as on the host gene expression.

To evaluate the effect of the viral infection on the host gene expression, *S. solfataricus* P2 (InF1) was infected to generate virus harbouring strains, i.e. SSV1-InF1, SSV2-InF1 and SSV1/SSV2-InF1. SSV1 and SSV2 show similar genome organization and gene synteny and, accordingly, SSV2 promoters were named after their SSV1 counterparts [9,26]. The only significant difference is the absence, in the SSV2 genome, of the SSV1 region involved in regulating the switch from the lysogenic to the UV-induced state. Microarray experiments were performed for all strains at two different growth phases, i.e. at 0.4 OD_{600nm} (early-exponential) and 1.2 OD_{600nm} (late-exponential), in order to define which viral genes were expressed. With this aim, microarray slides were co-hybridized with the total cDNA samples from:

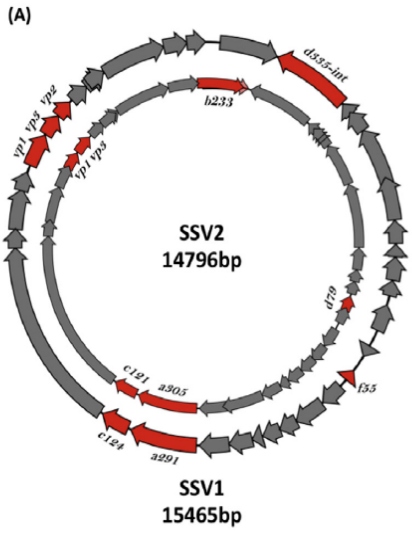
1) uninfected InF1 (ref) vs either SSV1-, SSV2-, or SSV1/SSV2-InF1 (exp) collected at 0.4 OD_{600nm}
2) uninfected InF1(ref) vs either SSV1-, SSV2-, or SSV1/SSV2-InF1 (exp) collected at 1.2 OD_{600nm}

cDNAs were prepared from the uninfected and infected strains and used as reference and experimental samples, respectively. SSV1 genes expressed at both growth phases analysed were those encoding: i) the structural proteins VP1, VP2 and VP3, ii) the integrase D335, iii) the transcription repressor F55 and iv) A291 and C124 (Fig. 1), for which virion docking/release and structural functions have been proposed, respectively [10,26]. Interestingly, most of viral genes expressed by the SSV2-InF1 strain were homologous to those expressed from SSV1, i.e.: VP1, VP3, A305 (SSV1-A291) and C121 (SSV1-C124) (Fig. 1). In addition, DnaA-like protein B233 and D79 were found expressed. A transcriptional activity was also detected by a probe matching the 3′ non-coding region of *b233* gene, thus indicating that the mRNA of this gene carries a long untranslated tail like the homolog on the pSSVx genome [9,35] (Fig. 1). In the double-infected strain (SSV1/SSV2-InF1), viral genes expressed were those encoding: i) VP1 and VP3 from both SSV1 and SSV2, ii) the homologous proteins A291/A305 and C124/C121, iii) F55 from SSV1 and iv) B233 and D79 from SSV2 (Fig. 1). Interestingly, gene expression patterns of SSV1 and SSV2 are nearly identical, despite the fact that replication induction for these two viruses is triggered by different stimuli, i.e. the UV-light exposure and the host physiological/metabolic state, respectively. Therefore, this analysis defined a minimal set of genes required in the carrier state for the replication and packaging of both fuselloviruses (Fig. 1).

In order to evaluate variation of the expression levels of viral genes during the host growth, the following co-hybridizations were carried out:

1) SSV1-InF1 (ref) collected at 0.4 OD_{600nm} vs SSV1-InF1 (exp) collected at 1.2 OD_{600nm}
2) SSV2-InF1 (ref) collected at 0.4 OD_{600nm} vs SSV2-InF1 (exp) collected at 1.2 OD_{600nm}
3) SSV1/SSV2-InF1 (ref) collected at 0.4 OD_{600nm} vs SSV1/SSV2-InF1 (exp) collected at 1.2 OD_{600nm}

cDNAs from infected strains collected at 0.4 OD_{600nm} and 1.2 OD_{600nm} were used as reference and experimental samples, respectively. This analysis revealed a constitutive expression of all



(B)

Viral genes expressed				
SSV1-InF1	SSV2-InF1	SSV1/SSV2-InF1		Function
SSV1-vp1	*SSV2-vp1*	*SSV1-vp1*	*SSV2-vp1*	Structural
SSV1-vp2	[a]H.N.D.	[c]N.E.	[a]H.N.D.	Structural
SSV1-vp3	*SSV2-vp3*	*SSV1-vp3*	*SSV2-vp3*	Structural
d335	[b]H.N.E.	[c]N.E.	[c]N.E.	Integrase
f55	[a]H.N.D.	*f55*	[a]H.N.D.	Transcription repressor
a291	*a305*	*a291*	*a305*	Putative viral docking/release
c124	*c121*	*c124*	*c121*	Putative structural
[b]H.N.E.	*b233*	[b]H.N.E.	*b233*	DnaA-like protein
[b]H.N.E.	*d79*	[b]H.N.E.	*d79*	Unknown

[a] Homologue not detected, [b] Homologue not expressed, [c] No expression

Fig. 1. Schematic representation of the SSV1 and SSV2 genomes. (A) Red-filled arrows are viral genes expressed in the carrier state for both SSV1 (outer) and the SSV2 (inner) genomes. Clockwise- and anticlockwise-oriented arrow heads represent ORFs encoded by the plus and minus strand, respectively. (B) List of viral genes expressed in all strains analysed.

Table 1
Number of up- and down-regulated genes and their functional distribution.

Functional group[a]	Gene expression							
	Up-regulated genes				Down-regulated genes			
	InF1	SSV1-InF1	SSV2-InF1	SSV1/SSV2-InF1	InF1	SSV1-InF1	SSV2-InF1	SSV1/SSV2-InF1
Amino acids biosynthesis	2	1	2	1	2	0	2	0
Cellular envelope and membrane	1	0	1	0	0	0	1	0
Cellular process	2	1	4	2	2	1	8	2
Central intermediary metabolism	0	2	0	2	0	0	2	0
Cofactor biosynthesis	0	0	0	0	0	0	2	1
Energy metabolism	10	1	19	0	1	0	12	3
Hypothetical protein[b]	16	3	10	1	6	4	16	3
IS elements	1	2	6	0	1	0	0	0
Lipid metabolism	2	0	3	1	0	0	0	0
Nucleotide metabolism	1	0	0	0	0	0	7	1
Protease and protein modification	0	0	0	0	1	0	2	0
Replication and repair	1	0	1	0	0	0	3	2
RNA	2	1	1	1	0	1	0	0
Transcription and regulation	3	1	11	2	5	2	7	2
Translation	0	0	0	0	7	4	14	0
Transport	17	4	7	1	1	1	5	2
Uncategorized/helicases	1	1	3	0	1	0	2	2
Cas genes	0	0	8	0	0	0	0	0
SSV2 genes[c]	0	0	0	0	0	0	1	1
STIV infection differentially regulated (Ortman et al., 2008)	1	0	1	0	0	0	1	0
Total	60	17	77	11	27	13	85	19

[a] Putative functions are derived from the website of the *Sulfolobus solfataricus* P2 complete sequencing project (http://www-archbac.u-psud.fr/projects/sulfolobus/).
[b] Hypothetical proteins with no putative function.
[c] The SSV2 down-regulated gene is *a305*.

the above-listed SSV1 and SSV2 genes, except for the SSV2 ORF a305, which was down-regulated (Table 1).

3.2. Gene expression analysis in the uninfected InF1 strain

To evaluate the effect of viral infection on host gene expression, we first identified up- and down-regulated genes during the growth of the uninfected InF1 strain. With this aim, microarray slides were co-hybridized with total cDNA samples as follows:

InF1 collected at 0.4 OD_{600nm} (ref) vs InF1 collected at 1.2 OD_{600nm} (exp)

A total of 60 up-regulated and 27 down-regulated genes were detected (Table 1). Among the up-regulated ones, expression of 38 genes increased by 2.0–4.9 folds, 13 showed a 5.0–9.9 fold-change and only for 5 genes expression was up-regulated between 10.0 and 33.0 folds. On the other hand, all down-regulated genes showed a fold of repression comprised in the range 2.0–4.9, except for *sso2574* whose transcription was down-regulated by 5.9 folds.

A remarkable number of the up-regulated *S. solfataricus* genes encode proteins belonging to three functional categories, i.e. hypothetical protein, transport and energy metabolism (Table 1). Transcriptional activation of genes involved in the transport of small metabolites is not surprising and it is likely due to the shortage of nutrients that occurs in a late stage of the growth. Moreover, it is interesting that a homolog of the gene encoding the bacitracin resistance protein (*sso1860*) is up-regulated, hinting to defence mechanisms acting against xenobiotic peptides produced by other member cells to face overpopulation. On the other hand, down-regulated genes mostly cluster into the categories of transcription and regulation as well as translation (Table 1). In particular, down-regulation of many genes encoding for ribosomal proteins is consistent with the reduction of protein synthesis and, in turn, with the decrease of cell growth rate. A complete list of the differentially regulated genes, grouped in functional categories, is reported in the Table S2.

3.3. SSV1 infection has a slight effect on the host gene expression

In order to detect remodelling of the host gene expression in the SSV1-InF1 lysogenic strain, a microarray analysis was carried out by co-hybridizing cDNA samples as follows:

SSV1-InF1 collected at 0.4 OD_{600nm} (ref) vs SSV1-InF1 collected at 1.2 OD_{600nm} (exp)

To reveal host genes whose expression is altered as a consequence of the SSV1 infection, we excluded from the analysis those that were up- and down-regulated in the uninfected InF1 strain as a consequence of the physiological changes occurring in the passage from the early to the late exponential growth phase. Among 30 differentially expressed host genes, 17 were up- and 13 down-regulated throughout the growth of the SSV1-InF1 strain (Table 1). Changes in the expression level tended to be higher for up-regulated genes. The majority of the down-regulated ones decreased by 2–2.5-folds, whereas 5 genes were up-regulated with an increase from 2.8 to 4.3-fold (Table S3). Although these genes are scattered over several functional categories, a similar trend to that of the uninfected strain was observed. Indeed, genes encoding proteins involved in the transport and translation are up- and down-regulated, respectively.

These data show that the infection by SSV1 has only a limited effect on the host gene expression, suggesting a harmonic coexistence with the host in the carrier state. Accordingly, SSV1 is a temperate virus that self-regulates its gene expression in the carrier state through the pleiotropic effect of the transcription regulator F55 [11,16], which represses the expression of the UV-inducible as well as of the early genes.

3.4. Remodelling of the host gene expression induced by SSV2 infection includes the CRISPR-Cas system activation

To investigate the variation of host gene expression during the growth of the stably infected SSV2-InF1 strain, a microarray analysis was carried out by co-hybridizing cDNA samples as follows:

SSV2-InF1 collected at 0.4 OD_{600nm} (ref) vs SSV2-InF1 collected at 1.2 OD_{600nm} (exp)

As already described for the SSV1-InF1 strain, all variations of gene expression reported herein for the SSV2-InF1 strain have to be considered merely due to the SSV2 infection. A total of 162 host genes were differentially expressed throughout the growth of the SSV2-InF1 strain, with 77 and 85 genes that were found to be up- and down-regulated, respectively (Table 1). By comparing the total number of differentially regulated host genes in SSV1-InF1 (30 genes) to that of SSV2-InF1 (162 genes), it is evident that the remodelling occurring as consequence of the SSV2 infection is remarkable (Table 1). Expression variation is in general higher for up-regulated genes (between 2.0 and 7.9 folds) than for those down-regulated (between 2.0 and 6.0 folds). Moreover, expression of the majority of the latter decreased by 3.0-folds or less, whereas 34 up-regulated genes were differentially regulated of 4.0-folds or greater (Table S4). Intriguingly, although SSV2 is a not-lytic virus, its effect on the host gene expression is comparable to the lytic STIV and SIRV2 [27,28]. Indeed, 5% of the *S. solfataricus* genes were directly affected by the SSV2 infection, among which, those encoding for proteins belonging to the functional categories of energy metabolism as well as transcription and regulation tended to be up-regulated (Table 1). Furthermore, transcriptional induction of IS elements (Tab. S4) has already been reported after SIRV2 infection [29], as consequence of UV-light exposure [10] and heat shock [36], thus highlighting their involvement in the *S. solfataricus* stress response. On the other hand, down-regulated genes are mostly associated with categories of translation (Table 1 and Table S4). Moreover, repression of stress response genes encoding for the proteasome subunit (*sso0278*) and Bcp3 (*sso0225*) resembles the response of *S. solfataricus* to the infection by SIRV2, which is able in this way to circumvent host defences [28]. Expression remodelling of genes involved in informational processing (i.e. transcription and translation) has been also observed after infection with STIV [27] and SIRV2 [28]. Accordingly, like the latter, SSV2 might take the control over the host informational machinery to replicate its own genome.

One of the most interesting outcomes from this analysis was the expression variation observed for a group of genes involved in CRISPR-Cas antiviral system, i.e.: i) the type IA interference cassette located downstream the locus C, which includes *sso1439* (cas3″), *sso1441* (cas5), *sso1442* (cas7) and *sso1443* (csa5); ii) *sso1424* (small subunit of CASCADE, CRISPR-associated Complex for Antiviral Defence) and *sso1425* (csm3) belonging to the type IIIA interference cassette localized downstream the former one; iii) *sso1389* (csx1 putative transcription factor) localized upstream the locus A and iv) *sso1997* (cas7 type IA) lying in the proximity of the locus F (Fig. 2, Table 2). In parallel, with the exception of the locus E, all CRISPR loci were found to be up-regulated during the growth of the SSV2-InF1 strain, with the highest expression induction observed for loci A, C and D (Table 2). Noteworthy, proteins encoded by the aforementioned up-regulated type IA interference cassette constitute the CASCADE effector complex of *S. solfataricus*, which is involved in the interference step of the defence system [31]. Interestingly, loci A, B, D and F of *S. solfataricus* P2 strain contain spacers matching fuselloviral genomes with the highest density observed at the leader-proximal region of the locus F [37]. Although this latter is a non-extending locus, due to the lack of a leader sequence, it can still be useful in challenging viral infection since it generates crRNAs. Accordingly, the aCASCADE (archaeal CASCADE) co-purified with crRNAs from all CRISPR loci [38] and probably exert its activity with all the crRNAs of *S. solfataricus*, including those from locus F [39]. Moreover, the expression of this locus was confirmed in the strain SSV2-InF1 by RT-PCR (data not shown).

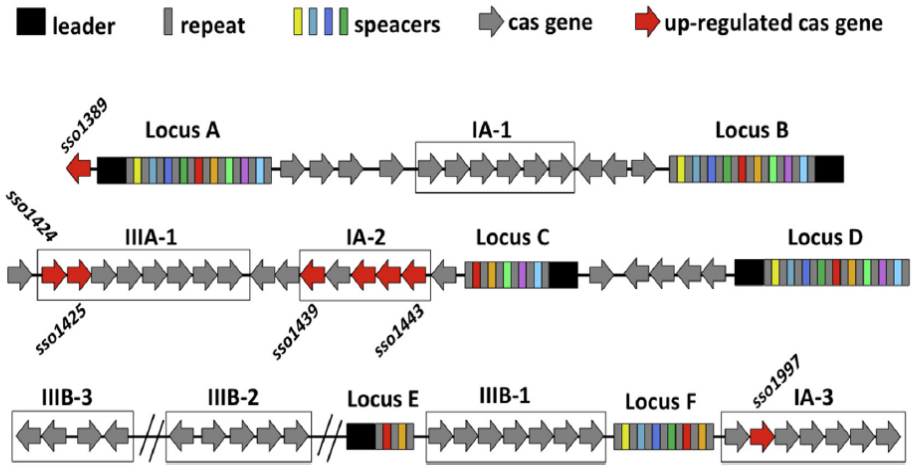


Fig. 2. CRISPR-Cas loci of *S. solfataricus* P2. The six CRISPR-Cas loci of the *S. solfataricus* P2 genome are schematized, where red-filled arrows are *cas* genes up-regulated in the SSV2-InF1 strain. Gene cassettes are black-framed and labelled.

3.5. The presence of SSV1 quenches the effect of SSV2 infection on the host gene expression

In order to evaluate variation of the host genes transcription caused by the co-presence of SSV1 and SSV2, hybridizations were carried out using cDNA samples as follows:

SSV1/SSV2-InF1 collected at 0.4 OD_{600nm} (ref) vs SSV1/SSV2-InF1 collected at 1.2 OD_{600nm} (exp)

As done before, data from the uninfected strain were used to filter those of the double-infected one, so that gene expression variation reported here is merely due to the presence of both viruses. As concerns viral genes, no significant variation was observed with the exception of the SSV2 gene *a305* that showed a down-regulation trend, similarly to that observed for the single-infected strain SSV2-InF1 (Table S5). On the other hand, among 30 differentially regulated host genes, 11 were up- and 19 down-regulated during the growth of the double-infected strain (Table 1). Therefore, the presence of SSV1 in the same host cells of SSV2 quenched the gene expression variation from 162 (being differentially regulated in the single-infected SSV2-InF1 strain) to only 30 genes. Surprisingly, genes and clusters of the CRISPR-Cas system were not induced in double-infected cells. Instead, the expression pattern of the viral genes is overall identical to that displayed by the single-infected SSV1- and SSV2-InF1 strains. The two fuselloviruses show syntenic genomes, with the only significant difference in the region responsible for the UV-inducibility of SSV1, which is lacking in the SSV2 genome [9]. Therefore, it is tempting to speculate that the SSV1 F55 transcription factor, encoded by this region, interferes with the remodelling of the host gene expression induced by SSV2.

Table 2
CRISPR loci and *cas* genes of *Sulfolobus solfataricus* P2 whose expression is affected by SSV2 infection.

Gene ID	Function
	Cas gene product
sso1389	*csx1* (CRISPR-Cas system-associated protein)
sso1424	*csa5* (CRISPR-Cas system-associated protein)
sso1425	*csm3* (CRISPR-associated RAMP)
sso1439	*cas*$^{\#}$ (nuclease subunits of Cas3)
sso1441	*cas5* (CASCADE complex core)
sso1442	*cas7* (CRISPR-associated protein, Csa2)
sso1443	csa5 (CRISPR-associated protein)
sso1997	CRISPR-associated auto-regulator, DevR family homolog
CRISPR locus	Probe position[a]
SSOLCTR-A	1708–1767
SSOLCTR-A	3373–3432
SSOLCTR-B	STIV
SSOLCTR-B	278–343
SSOLCTR-B	1493–1557
SSOLCTR-C	pNOB8
SSOLCTR-C	10–71
SSOLCTR-C	1916–1980
SSOLCTR-D	ATV
SSOLCTR-D	105–174
SSOLCTR-D	483–551
SSOLCTR-D	5568–5630
SSOLCTR-F	3689–3753
SSOLCTR-F	4350–4419

[a] Spacer position in each locus. STIV, pNOB8 and ATV are probes matching spacers against these genetic elements.

3.6. Isolation of single clones from the CRISPR-Cas responsive SSV2-InF1 strain

To study if the activation of the CRISPR-Cas system enabled the cells to get rid of SSV2, we performed a screening of the SSV2-InF1 strain to isolate cured cells. Therefore, clones showing a decreased viral titre, compared to the SSV2-InF1 initial population, were further characterized at DNA level to evaluate SSV2 content through semi-quantitative PCR. Results are shown for clones 1, 1Q and $1Q_1$ that are representative of the first, second and third round of selection, respectively. The PCR products were analysed for each sample on agarose gel at the 20th, 25th and 30th cycle of amplification and the initial population SSV2-InF1 was used as reference (Fig. 3). Although the intensity of the *orc1* signal is nearly identical when the same amplification cycle is considered, the signal relative to the viral gene *vp3* progressively decreases until it becomes hardly visible for the clone $1Q_1$ (Fig. 3). Therefore, the low viral titre observed for this latter clone (two orders of magnitude less than the SSV2-InF1 initial population), is mirrored by a comparable drop of the intracellular SSV2 DNA content (Fig. 3).

In parallel, a PCR analysis of the SSV2 integration site was carried out for the same clones using the primers SSV2attApO1, SSV2attApO2 and SSV2attApO3 [25]. The primer couple pO1–pO2 allows the amplification of an approximately 968 bp fragment if the integration site is empty (no provirus). On the other hand, the couple ApO1–ApO3 leads to the amplification of a 722 bp product only when the integration site is occupied by SSV2 (Fig. 4). Whereas

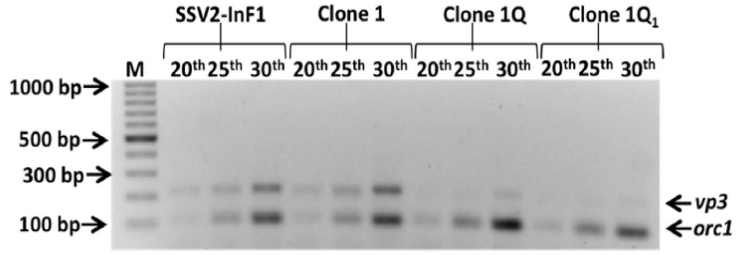


Fig. 3. SSV2 DNA detection by semi-quantitative PCR. Black-straight arrows point out to molecular size markers as well as to host (*orc1*) and viral (*vp3*) PCR products. Total DNA samples were prepared from the initial population (SSV2-InF1) as well as from the isolated clones (1, 1Q and $1Q_1$). The decreased intensity of the *vp3* signals indicates that the SSV2 copy number dropped throughout the isolation procedure.

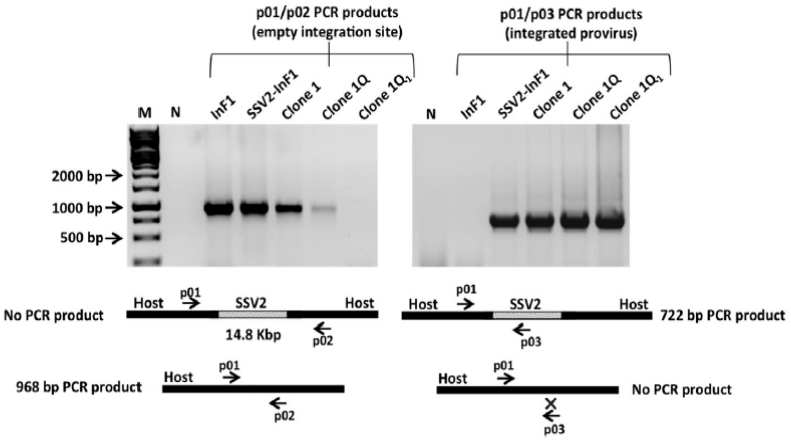


Fig. 4. PCR analysis of the SSV2 integration site occupancy. Black-straight arrows point out to molecular size markers. To check the occupancy of the SSV2 integration site, PCR products were amplified using the pO1/pO2 (968 bp, free integration site) and pO1/pO3 (722 bp, integrated provirus) primer couples from the SSV2-InF1 as well as from the isolated clones (1, 1Q and 1Q). As shown in the right panel, all infected clones carry the provirus. However, provirus-free cells still persist in the cultures (left panel) although their amount decreases throughout the isolation procedure. Eventually, no empty integration sites were detected in the $1Q_1$ clone.

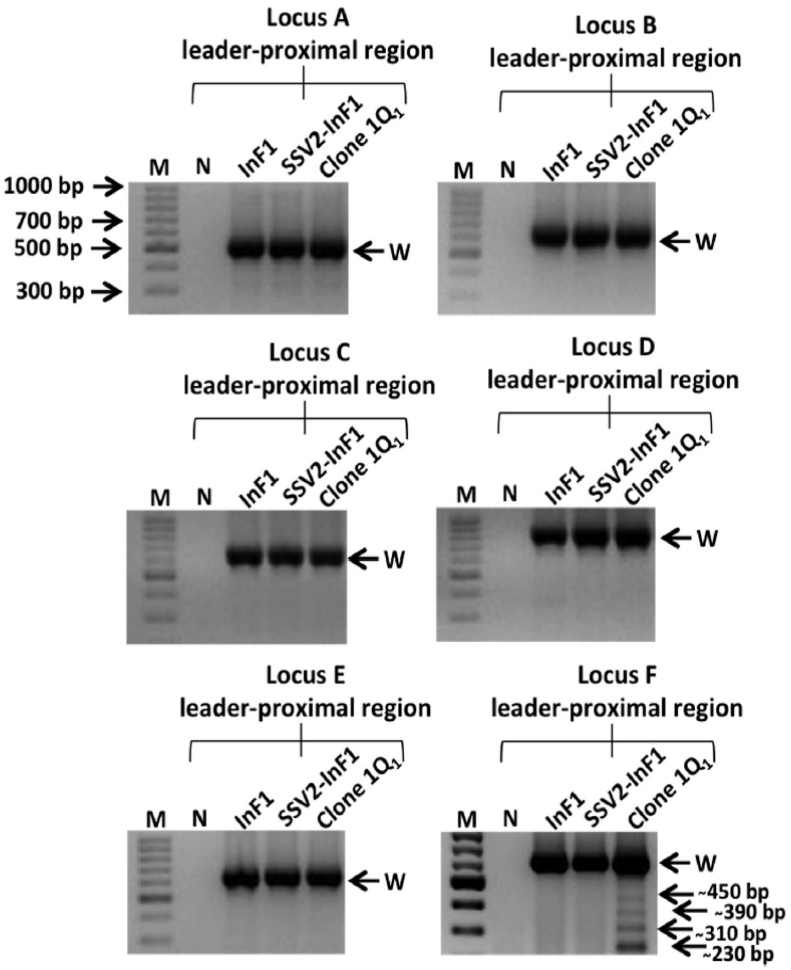


Fig. 5. Leader-proximal region amplification of the *S. solfataricus* P2 CRISPR array. PCRs were performed to amplify leader-proximal regions of all six CRISPR loci (A–F) from the virus-free strain (InF1), SSV2-infected initial population (SSV2-InF1) and the $1Q_1$ clone. The wild-type PCR product (W) has been amplified for all loci, thus indicating the lack of spacer acquisition (no adaptation). Intriguingly, shorter PCR products were amplified from the locus F of the clone $1Q_1$. Sequencing of these fragments confirmed loss of repeat/spacer units from this locus (see Fig. 6). M and N stay for marker and negative control, respectively.

amplification of the product pO1–pO2 for the infected strain SSV2-InF1 as well as for the clone 1 and 1Q indicates that a fraction of the cell population does not carry the provirus, the absence of such amplicon in the $1Q_1$ clone suggests that virtually all cells carry the integrated SSV2 in the attachment site. The amplification of the product pO1–pO3 further confirms the presence of the provirus (Fig. 4). Since the excision of the provirus strictly depends on the presence of active integrase/excision enzymes, encoded by the episomal copies [40], degradation of the latter by the host CRISPR-Cas system leads to the entrapment of the provirus (Fig. 4), as already hypothesized elsewhere [41].

3.7. SSV2 infection causes deletions at the leader-proximal region of the locus F

A fundamental step of the CRISPR-Cas system is the adaptation, which consists in the Cas proteins-mediate incorporation of new spacers from foreign DNAs. This occurs immediately downstream the leader sequence of a given CRISPR locus upon viral infection. In order to detect the integration of new spacers, leader-proximal regions of all *S. solfataricus* loci were amplified (Fig. 5). In particular, primer couples were chosen as described elsewhere [33], so that integration of new spacers would have produced longer PCR products when compared with a control sample (uninfected InF1).

Our PCR analysis detected no differences in the length of the amplified products (loci A–E) among the initial population SSV2-InF1 and the isolated clones (Fig. 5), thus confirming that no adaptation occurred upon infection with SSV2 at these loci. Nevertheless, smaller PCR products (between 230 and 450 bp) were found when the leader-proximal region of the CRISPR locus F was amplified from the $1Q_1$ DNA (Fig. 5). In order to shed light on the identity of these amplicons, PCR products were run on an agarose gel and single bands were cut off, purified and sequenced. Surprisingly, this analysis showed that progressive deletions occurred at the leader-proximal region of the locus F and up to six repeat-spacer units were found to be lacking (Fig. 6).

In this regard, it is noteworthy that Stern and co-workers have hypothesized that the incorporation of self-targeting spacers might lead to autoimmunity and select for the loss of CRISPR functions [42]. Moreover, it has been demonstrated that when *S. solfataricus* is transformed with a plasmid that carries a gene essential for the host and which is also a target of the CRISPR system, surviving transformants show mutations that eliminate the plasmid-targeting spacer [32]. What would happen if the invading DNA, such as a virus, could not be destroyed because it managed to integrate into the host genome, thus becoming 'self'? In the hypothesis that the host is challenging the SSV2 infection (as shown by microarray data), it is likely that the CRISPR-Cas system targets also the integrated viral genome (i.e., the provirus). This event casts a huge fitness cost on cells harbouring self-targeting spacers [43]. As a consequence, the selective pressure drives the inactivation of the CRISPR-Cas system to ensure the survival of SSV2-lysogens. Our results indicate that this is achieved through deletion of spacer units responsible for the recognition of the SSV2 genome (Figs. 5 and 6).

4. Conclusions

Analysis of both viral and host transcriptomes upon SSV1 and SSV2 infection in *S. solfataricus* has revealed new insights into archaeal host–virus interactions. Previous studies were carried out to detect variation of viral and host gene expression in the early phases of the SSV2 infection [26], upon UV-irradiation for SSV1 [10] or on lytic viruses [27–29]. Therefore, the dissection of the viral transcription patterns during the carrier state has never been performed. Herein, by exploiting stably infected cells in the absence of any stimuli, we demonstrate not only that the host response towards the two viruses is extremely different, but that it is also

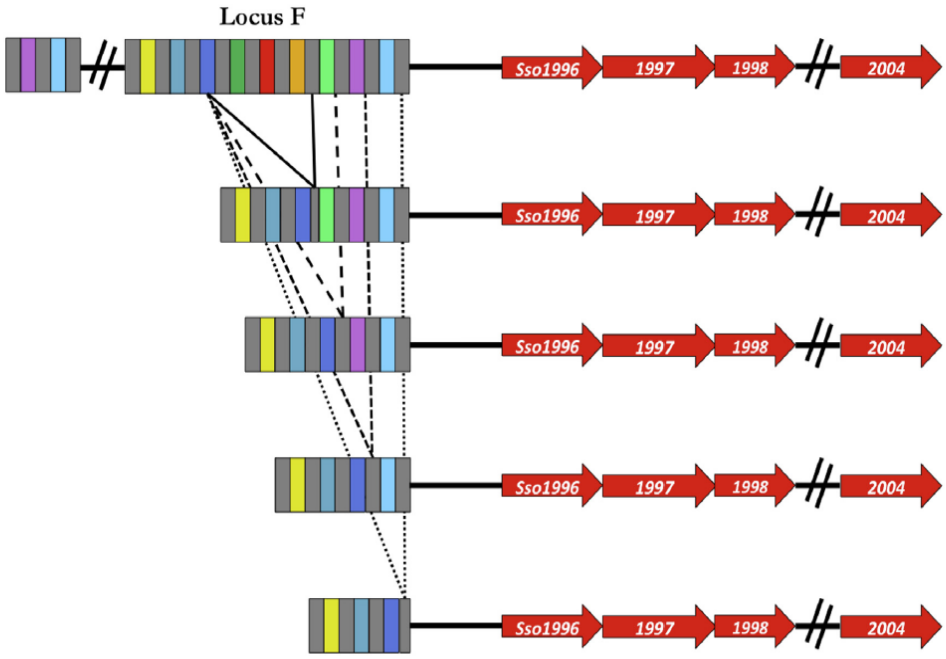


Fig. 6. Schematic illustration of repeat/spacer units loss from the locus F. The array is reported as grey rectangles (repeats) interspersed by colourful rectangles (spacers). A short intergenic region separates the array from the opposite-oriented *cas* genes cassette (IA3, see also Fig. 2). The four different loci F with deletions are schematised to show the loss of 2–6 repeat/spacer units in the clone $1Q_1$.

influenced by the co-presence of both fuselloviruses (Table 1). In particular, whereas *S. solfataricus* establishes a harmonious coexistence with SSV1, the reaction against the infection by SSV2 is remarkable and includes the transcriptional activation of CRISPR loci and *cas* genes (Table 2). This result is consistent with the isolation of single clones showing a low SSV2 copy number (Fig. 3). Our study highlights an interesting aspect of the host–virus interaction in the frame of the CRISPR response, i.e. host cells containing an integrated provirus (Fig. 4) are forced to develop a survival strategy in order to avoid self-attack by the CRISPR-Cas system. In fact, since the provirus is identical in sequence to the episomal copies, it can be targeted by the CRISPR-Cas system as well, thus casting a fitness cost on cells that are actively challenging the SSV2 infection. Our data show, for the first time, that *S. solfataricus* cells developed a specific strategy to safeguard host genome integrity, i.e. throughout deletion of self-targeting spacers (Figs. 5 and 6). How deletion of specific spacers at CRISPR loci occurs is matter of further investigation and it is expected to reveal completely novel molecular components and/or mechanisms of the CRISPR-Cas system.

Another relevant aspect highlighted by this study is the absence of the CRISPR-Cas response in the double-infected SSV1/SSV2-InF1 strain (Table 1 and Table S5). The two viral genomes are overall identical with the only significant difference being the absence in the SSV2 genome of a region responsible for the UV-induction in SSV1 (Fig. 1). Therefore, it is tempting to speculate that transcription factors and/or other molecular components encoded by this SSV1 region are responsible for silencing the CRISPR-Cas response in the double-infected strain. A combination of biochemical and genetic approaches is needed to move toward a better understanding of the mutual influence of the two fuselloviruses in this virus–host system.

Conflict of interest

No conflict of interest.

Acknowledgements

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Appendix A. Supplementary material

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.biochi.2015.04.006.

References

[1] C.A. Suttle, Marine viruses – major players in the global ecosystem, Nat. Rev. Microbiol. 5 (2007) 801–812.
[2] A.M. Comeau, G.F. Hatfull, H.M. Krisch, D. Lindell, N.H. Mann, D. Prangishvili, Exploring the prokaryotic virosphere, Res. Microbiol. 159 (2008) 306–313.
[3] M.K. Pietilä, T.A. Demina, N.S. Atanasova, H.M. Oksanen, D.H. Bamford, Archaeal viruses and bacteriophages: comparisons and contrasts, Trends Microbiol. 22 (2007) 334–344.
[4] D. Prangishvili, The wonderful world of archaeal viruses, Annu. Rev. Microbiol. 8 (2013) 565–585.
[5] H. Wang, N. Peng, S.A. Shah, L. Huang, Q. She, Archaeal extrachromosomal genetic elements, Microbiol. Mol. Biol. Rev. 79 (2015) 117–152.
[6] M. Pina, A. Bize, P. Forterre, D. Prangishvili, The archeoviruses, FEMS Microbiol. Rev. 35 (2011) 1035–1054.
[7] X. Peng, R.A. Garrett, Q. She, Archaeal viruses – novel, diverse and enigmatic, Sci. China Life Sci. 55 (2012) 422–433.
[8] M. Krupovic, E.R. Quemin, D.H. Bamford, P. Forterre, D. Prangishvili, Unification of the globally distributed spindle-shaped viruses of the *Archaea*, J. Virol. 88 (2013) 2354–2358.
[9] P. Contursi, S. Fusco, R. Cannio, Q. She, Molecular biology of fuselloviruses and their satellites, Extremophiles 3 (2014) 473–489.
[10] S. Fröls, P.M. Gordon, M.A. Panlilioc, C. Schleper, C.W. Sensen, Elucidating the transcription cycle of the UV-inducible hyperthermophilic archaeal virus SSV1 by DNA microarrays, Virology 365 (2007) 48–59.
[11] S. Fusco, Q. She, S. Bartolucci, P. Contursi, T_{lys}, a newly identified *Sulfolobus* spindle-shaped virus 1 transcript expressed in the lysogenic state, encodes a DNA-binding protein interacting at the promoters of the early genes, J. Virol. 87 (2013) 5926–5936.
[12] W.D. Reiter, P. Palm, W. Zillig, Analysis of transcription in the archaebacterium *Sulfolobus* indicates that archaebacterial promoters are homologous to eukaryotic pol II promoters, Nucleic Acids Res. 16 (1988) 1–19.
[13] W.D. Reiter, P. Palm, W. Zillig, Transcription termination in the archaebacterium *Sulfolobus*: signal structures and linkage to transcription initiation, Nucleic Acids Res. 16 (1988) 2445–2459.
[14] M. Nadal, G. Mirambeau, P. Forterre, W.D. Reiter, M. Duguet, Positively supercoiled DNA in a virus-like particle of an archaebacterium, Nature 321 (1986) 256–258.
[15] C. Schleper, K. Kubo, W. Zillig, The particle SSV1 from the extremely thermophilic archaeon *Sulfolobus* is a virus: demonstration of infectivity and of transfection with viral DNA, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 7645–7649.
[16] S. Fusco, Q. She, G. Fiorentino, S. Bartolucci, P. Contursi, Unravelling the role of the F55 regulator in the transition from lysogeny to UV induction of *Sulfolobus* spindle-shaped virus, J. Virol. 89 (2015) [in press].
[17] P. Kraft, D. Kümmel, A. Oeckinghaus, G.H. Gauss, B. Wiedenheft, M. Young, C.M. Lawrence, Structure of D-63 from *Sulfolobus* spindle-shaped virus 1: surface properties of the dimeric four-helix bundle suggest an adaptor protein function, J. Virol. 78 (2004) 7438–7442.
[18] P. Kraft, A. Oeckinghaus, D. Kümmel, G.H. Gauss, J. Gilmore, B. Wiedenheft, M. Young, C.M. Lawrence, Crystal structure of F-93 from *Sulfolobus* spindle-shaped virus 1, a winged-helix DNA binding protein, J. Virol. 78 (2004) 11544–11550.
[19] S.K. Menon, W.S. Maaty, G.J. Corn, S.C. Kwok, B.J. Eilers, P. Kraft, E. Gillitzer, M.J. Young, B. Bothner, C.M. Lawrencem, Cysteine usage in *Sulfolobus* spindle-shaped virus 1 and extension to hyperthermophilic viruses in general, Virology 376 (2008) 270–278.
[20] F. Guillière, N. Peixeiro, A. Kessler, B. Raynal, N. Desnoues, J. Keller, M. Delepierre, D. Prangishvili, G. Sezonov, J.I. Guijarro, Structure, function, and targets of the transcriptional regulator SvtR from the hyperthermophilic archaeal virus SIRV1, J. Biol. Chem. 284 (2009) 22222–22237.
[21] P. Contursi, K. D'Ambrosio, L. Pirone, E. Pedone, T. Aucelli, Q. She, G. De Simone, S. Bartolucci, C68 from the *Sulfolobus islandicus* plasmid–virus pSSVx is a novel member of the AbrB-like transcription factor family, Biochem. J. 435 (2011) 157–166.
[22] C. Schlenker, A. Goel, B.P. Tripet, S. Menon, T. Willi, M. Dlakić, M.J. Young, C.M. Lawrence, V. Copié, Structural studies of E73 from a hyperthermophilic archaeal virus identify the "RH3" domain, an elaborated ribbon–helix–helix motif involved in DNA recognition, Biochemistry 51 (2012) 2899–2910.
[23] P. Contursi, S. Fusco, D. Limauro, G. Fiorentino, Host and viral transcriptional regulators in *Sulfolobus*: an overview, Extremophiles 17 (2013) 881–895.
[24] P. Contursi, B. Farina, L. Pirone, S. Fusco, L. Russo, S. Bartolucci, R. Fattorusso, E. Pedone, Structural and functional studies of Stf76 from the *Sulfolobus islandicus* plasmid–virus pSSVx: a novel peculiar member of the winged helix-turn-helix transcription factor family, Nucleic Acids Res. 42 (2014) 5993–6011.
[25] P. Contursi, S. Jensen, T. Aucelli, M. Rossi, S. Bartolucci, Q. She, Characterization of the *Sulfolobus* host–SSV2 virus interaction, Extremophiles 10 (2006) 615–627.
[26] Y. Ren, Q. She, L. Huang, Transcriptomic analysis of the SSV2 infection of *Sulfolobus solfataricus* with and without the integrative plasmid pSSVi, Virology 441 (2013) 126–134.
[27] A.C. Ortmann, S.K. Brumfield, J. Walther, K. McInnerney, S.J. Brouns, H.J. van de Werken, B. Bothner, T. Douglas, J. van de Oost, M.J. Young, Transcriptome analysis of infection of the archaeon *Sulfolobus solfataricus* with *Sulfolobus* turreted icosahedral virus, J. Virol. 82 (2008) 4874–4883.
[28] E. Okutan, L. Deng, S. Mirlashari, K. Uldahl, M. Halim, C. Liu, R.A. Garrett, Q. She, X. Peng, Novel insights into gene regulation of the rudivirus SIRV2 infecting *Sulfolobus* cells, RNA Biol. 10 (2013) 875–885.
[29] T.E. Quax, M. Voet, O. Sismeiro, M.A. Dillies, B. Jagla, J.Y. Coppée, G. Sezonov, P. Forterre, J. van der Oost, R. Lavigne, D. Prangishvili, Massive activation of archaeal defense genes during viral infection, J. Virol. 87 (2013) 8419–8428.
[30] R.M. Ceballos, C.D. Marceau, J.O. Marceau, S. Morris, A.J. Clore, K.M. Stedman, Differential virus host-ranges of the *Fuselloviridae* of hyperthermophilic *Archaea*: implications for evolution in extreme environments, Front. Microbiol. 3 (2012) 295.

[31] J. Zhang, M.F. White, Hot and crispy: CRISPR-Cas systems in the hyperthermophile *Sulfolobus solfataricus*, Biochem. Soc. Trans. 41 (2013) 1422–1426.
[32] S. Gudbergsdottir, L. Deng, Z. Chen, J.V.K. Jensen, L.R. Jensen, Q. She, R.A. Garrett, Dynamic properties of the *Sulfolobus* CRISPR/Cas and CRISPR/Cmr systems when challenged with vector-borne viral and plasmid genes and protospacers, Mol. Microbiol. 79 (2011) 35–49.
[33] S. Erdmann, R.A. Garrett, Selective and hyperactive uptake of foreign DNA by adaptive immune systems of an archaeon via two distinct mechanisms, Mol. Microbiol. 85 (2012) 1044–1056.
[34] S. Fusco, M. Aulitto, S. Bartolucci, P. Contursi, A standardized protocol for the UV induction of *Sulfolobus* spindle-shaped virus 1, Extremophiles 19 (2015) 539–546.
[35] P. Contursi, R. Cannio, Q. She, Transcription termination in the plasmid/virus hybrid pSSVx from *Sulfolobus islandicus*, Extremophiles 14 (2010) 453–463.
[36] C.R. Cooper, A.J. Daugherty, S. Tachdjian, P.H. Blum, R.M. Kelly, Role of vapBC toxin–antitoxin loci in the thermal stress response of *Sulfolobus solfataricus*, Biochem. Soc. Trans. 37 (2009) 123–126.
[37] R.K. Lillestøl, S.A. Shah, K. Brügger, P. Redder, H. Phan, J. Christiansen, R.A. Garrett, CRISPR families of the crenarchaeal genus *Sulfolobus*: bidirectional transcription and dynamic properties, Mol. Microbiol. 72 (2009) 259–272.
[38] N.G. Lintner, M. Kerou, S.K. Brumfield, S. Graham, H. Liu, J.H. Naismith, M. Sdano, N. Peng, Q. She, V. Copié, M.J. Young, M.F. White, C.M. Lawrence, Structural and functional characterization of an archaeal clustered regularly interspaced short palindromic repeat (CRISPR)-associated complex for antiviral defense (CASCADE), J. Biol. Chem. 286 (2011) 21643–21656.
[39] A. Manica, C. Schleper, CRISPR-mediated defense mechanisms in the hyperthermophilic archaeal genus *Sulfolobus*, RNA Biol. 10 (2013) 671–678.
[40] Q. She, X. Peng, W. Zillig, R.A. Garrett, Gene capture events in archaeal chromosomes, Nature 409 (2001) 478.
[41] R.A. Garrett, S.A. Shah, G. Vestergaard, L. Deng, S. Gudbergsdottir, C.S. Kenchappa, S. Erdmann, Q. She, CRISPR-based immune systems of the *Sulfolobales*: complexity and diversity, Biochem. Soc. Trans. 39 (2011) 51–57.
[42] A. Stern, L. Keren, O. Wurtzel, G. Amitai, R. Sorek, Self-targeting by CRISPR: gene regulation or autoimmunity? Trends Genet. 26 (2010) 335–340.
[43] M. Dyall-Smith, Dangerous weapons: a cautionary tale of CRISPR defence, Mol. Microbiol. 79 (2011) 3–6.

Supplementary material

Table S1. Primers used for PCR analyses.

Name	Sequence (5'-3')	Length (nt)
Lpr-LcsA-fw	AGCTTCTGACCCGCTCCTGA	20
Lpr-LcsA-rv	GCACATCATCAAACAATGGTAAGCC	25
Lpr-LcsB-fw	AGGGGTTTGTGGGATGGGTTGTG	23
Lpr-LcsB-rv	ACAACTACCACCACTACCACGG	22
Lpr-LcsC-fw	TCGCTTATCTCTCTCATGCGCCATT	25
Lpr-LcsC-rv	TGTCCCGTTTTTGTAAGTGGGGG	21
Lpr-LcsD-fw	AGTTCCACCCCCGAAGCTCCT	21
Lpr-LcsD-rv	AGCCGGGACAAGTTTCACAAATTGA	25
Lpr-LcsE-fw	ATAGGGAAAGAGTTCCCCCG	20
Lpr-LcsE-rv	TGACTCTAGTGCAATCTTCGA	21
Lpr-LcsF-fw	CGGCGTTATAATGGGTATCGGAATCGG	27
Lpr-LcsF-rv	GCTCACTATCTCACCCCTATCAATACCC	28
SSV2attAp01	GTGTTCTACCTTTTCCACAGTC	22
SSV2attAp02	TGGGTACGTCATTTATTGATCTT	23
SSV2attAp03	GCTTTTATGCAGTTATTGCTTT	22
orc1-fw	TATAAATTGTTATAGACATAGAACGCTGTA	30
orc1-rv	TTAAATACTTCTTGTGCCGATAGTCC	26
vp3-fw	GTTATTGGTGTAGTTCTGTT	20
vp3-rv	GGGTACAACAATTAAGACTA	20

Table S2. Uninfected *S. solfataricus* InF1 strain: a complete list of the up-regulated and down-regulated genes throughout the growth.

Gene ID	Average log2 Ratio	S.D. log2 Ratio	VGEF	Function
NT02SS2357	2.89	0.71	7.43	Intergenic region
SSO0127	1.07	0.17	2.09	2-isopropylmalate synthase
SSO0389	2.24	0.28	4.73	Glycosylated S-layer protein
SSO0488	2.06	1.23	4.18	Phosphate ABC transporter
SSO0489	5.06	0.40	33.46	Phosphate binding periplasmic protein
SSO0490	3.87	0.40	14.64	Phosphate transport system permease
SSO0491	2.51	1.13	5.71	Phosphate transport system permease
SSO0545	2.47	1.20	5.54	Hypothetical protein
SSO0786	2.54	0.83	5.80	Amino acid specific permease
SSO0929	1.56	0.32	2.94	Ribonucleotide reductase (nrd)
SSO1005	1.41	0.59	2.65	Hypothetical protein
SSO10788	2.03	0.12	4.09	Bacterial response and virulence
SSO1092	2.18	1.10	4.54	Hypothetical protein
SSO11071	1.08	0.12	2.12	Pyruvate synthase delta chain
SSO1129	1.74	0.41	3.35	Heterodisulfite reductase, subunit B
SSO1171	1.10	0.14	2.14	Maltose ABC transporter
SSO1183	2.76	1.39	6.76	Inorganic phosphate transporter
SSO11855	1.21	0.34	2.32	STIV-infection differentially regulated
SSO1227	2.03	0.90	4.07	Toluene-4-monooxygenase system protein A
SSO1237	1.14	0.11	2.21	First ORF in transposon ISC1491
SSO1287	1.70	0.64	3.25	Hypothetical protein
SSO1288	2.82	0.79	7.08	Hypothetical protein
SSO1320	1.18	0.18	2.27	Hypothetical protein
SSO1526	1.58	0.86	3.00	Pyruvate dehydrogenase, alfa subunit
SSO1665	1.34	0.54	2.54	Cytosine permease
SSO1835	2.40	1.29	5.26	DNA polymerase beta domain
SSO1858	1.74	0.65	3.34	Hypothetical protein
SSO1860	1.47	0.50	2.77	Bacitracin resistance protein
SSO1865	1.32	0.48	2.49	Universal stress protein family
SSO1894	2.73	0.69	6.64	Hypothetical protein
SSO1910	1.87	0.30	3.66	Hypothetical protein
SSO1931	2.07	1.11	4.20	Hypothetical protein
SSO2029	3.00	0.92	7.99	Hypothetical protein
SSO2044	1.71	0.41	3.27	NAD specific glutamate dehydrogenase
SSO2126	1.69	0.60	3.23	L-lactate permease
SSO2476	1.26	0.01	2.40	Metabolite permease, putative
SSO2505	3.49	0.92	11.24	Sugar transport protein
SSO2523	2.35	0.74	5.09	Long-chain-fatty-acid--CoA ligase
SSO2629	2.03	0.44	4.08	Oxidoreductase (flavoprotein)
SSO2630	1.17	0.38	2.25	SirA family protein
SSO2632	1.44	0.38	2.71	Hypothetical protein

SSO2636	2.27	0.67	4.83	Carbon monoxide dehydrogenase
SSO2756	1.38	0.17	2.60	Pyruvate synthase beta chain
SSO2797	1.18	0.34	2.27	Conserved hypothetical protein
SSO2815	1.26	0.27	2.39	2-oxoacid--ferredoxin oxidoreductase
SSO2863	1.82	1.01	3.53	Acetyl-CoA synthetase
SSO2864	1.29	0.09	2.45	Conserved hypothetical protein
SSO2966	1.36	0.59	2.57	Conserved hypothetical protein
SSO2967	3.04	0.32	8.20	Hypothetical protein
SSO2969	1.53	0.37	2.89	Quinol oxidase-2, subunit I
SSO2970	2.23	0.76	4.69	Quinol oxidase-2, cytochrome b
SSO2971	1.90	0.57	3.72	Quinol oxidase-2, rieske iron-sulfur protein-2
SSO2973	2.61	0.25	6.11	Quinol oxidase-2, subunit I/III, cytochrome aa3
SSO3043	3.35	0.43	10.19	ABC transporter, binding protein
SSO3053	4.67	2.27	25.53	Maltose ABC transporter
SSO3058	2.08	1.66	4.24	Maltose ABC transporter,
SSO3120	3.28	0.46	9.71	Metabolite transport protein, putative
SSO3180	2.42	0.03	5.33	Phosphate transporter related protein
SSO7127	1.79	0.71	3.46	non-coding RNA homolog
SSO0091	-2.22	0.69	-4.67	LSU ribosomal protein L7AE (rpl7AE)
SSO0164	-1.58	0.29	-2.99	LSU ribosomal protein S8E (rps8E)
SSO0200	-1.29	0.46	-2.44	ArsR family transcriptional regulators homolog
SSO0219	-1.55	0.63	-2.93	SSU ribosomal protein S12AB (rpS12AB)
SSO0278	-1.25	0.38	-2.38	Proteasome subunit
SSO0366	-1.49	0.49	-2.81	Glutamine synthetase
SSO0536	-1.86	0.46	-3.64	S-adenosylmethionine decarboxylase
SSO0742	-1.18	0.26	-2.26	LSU ribosomal protein L15E
SSO0746	-1.17	0.14	-2.26	SSU ribosomal protein S3AE (rps3AE)
SSO0752	-1.24	0.28	-2.37	LSU ribosomal protein L21E (rpl21E)
SSO0962	-1.71	0.39	-3.27	DNA binding protein SSO10b (Alba 1)
SSO1067	-1.34	0.44	-2.54	Second ORF in transposon ISC1359
SSO1100	-1.20	0.33	-2.30	Hypothetical protein
SSO1101	-1.39	0.54	-2.62	Transcriptional regulator
SSO1107	-1.28	0.39	-2.43	Hypothetical protein
SSO1586	-1.65	0.36	-3.13	Conserved hypothetical protein
SSO1593	-2.13	1.10	-4.38	Benzoate transporter
SSO2574	-2.56	0.84	-5.91	Ferredoxin (zfx-1)
SSO2595	-1.07	0.22	-2.10	Conserved hypothetical protein
SSO2613	-1.68	0.04	-3.21	Peroxiredoxin, bacterioferritincomigratory
SSO3155	-1.99	1.04	-3.98	Tryptophan repressor binding protein (wrbA)
SSO5343	-1.02	0.09	-2.02	Conserved hypothetical protein
SSO5410	-1.06	0.05	-2.08	Small nuclear riboprotein protein (snRNP-1)
SSO6454	-1.05	0.12	-2.08	snRNP-2 small nucleare riboprotein
SSO7114	-1.06	0.11	-2.08	SSU ribosomal protein S27E
SSO9724	-1.00	0.11	-2.00	Hypothetical protein

Table S3. *S. solfataricus* SSV1-InF1 infected strain: a complete list of the up-regulated and down-regulated genes throughout the growth.

Gene ID	Average log2 Ratio	S.D. log2 Ratio	VGEF	Function
small-RNA sR116	1.22	0.31	2.33	small cytoplasmic RNA
SSO0269	1.39	0.54	2.61	GTP-binding protein (hflX)
SSO0276	1.40	0.42	2.64	Small nuclear ribonucleoprotein (snRNP)
SSO0794	1.33	0.10	2.51	Transposase ISC1476
SSO0999	1.45	0.63	2.73	ABC Transporter
SSO10452	2.07	0.67	4.20	Hypothetical protein
SSO1052	1.38	0.39	2.60	Oligosaccharyltransferase STT3
SSO1069	1.85	0.77	3.62	Amino acid transporter
SSO1126	1.34	0.20	2.54	ChainA, Putative Oxidoreductase
SSO1284	1.56	0.36	2.94	Oligo/dipeptide transport, permease protein
SSO1717	1.47	0.66	2.76	Second ORF in transposon ISC1048
SSO1752	1.02	0.02	2.02	Hypothetical protein
SSO2660	1.39	0.55	2.61	Rieske iron-sulfur protein-1 (soxL-1)
SSO2661	1.50	0.29	2.83	P-aminobenzoate N-oxygenase
SSO3085	1.15	0.36	2.22	Hypothetical sulphur transporter
SSO3123	0.93	0.05	1.91	Hypothetical protein
SSO3211	1.02	0.03	2.03	4-aminobutyrate aminotransferase
small-RNA sR102	-1.27	0.53	-2.42	small cytoplasmic RNA
SSO0221	-1.30	0.15	-2.47	LSU ribosomal protein L30E (rpl30E)
SSO0637	-1.74	0.65	-3.34	Hypothetical protein
SSO0961	-1.16	0.23	-2.24	Hypothetical protein
SSO12181	-0.96	0.08	-1.94	Hypothetical protein
SSO2138	-1.53	0.33	-2.89	PadR family transcripitonal regulator
SSO2180	-1.35	0.49	-2.55	Hypothetical protein
SSO2253	-1.12	0.03	-2.18	Rubrerythrin (oxidative stress tolerance)
SSO2381	-0.92	0.05	-1.90	Translation initiation factor 2, beta subunit
SSO2827	-1.09	0.21	-2.13	Predicted transcriptional regulator
SSO5478	-1.05	0.21	-2.07	LSU ribosomal protein L24E (rpl24E)
SSO5663	-1.18	0.25	-2.26	Protein transport protein sec61
SSO5668	-0.98	0.13	-1.97	LSU ribosomal protein LX

Table S4. *S. solfataricus* SSV2-InF1 infected strain: a complete list of the up-regulated and down-regulated genes throughout the growth.

Gene ID	Average log2 Ratio	S.D. log2 Ratio	VGEF	Function
small-RNA_sR103	1.03	0.10	2.05	RNA
SSO0044	1.29	0.12	2.45	Heme bearing subunit I of the terminal oxidase (doxB)
SSO0045	1.32	0.07	2.50	Terminal oxidase, subunit (doxC)
SSO0227	1.40	0.17	2.63	DNA-directed RNA polymerase, subunit B'' (rpoB1)
SSO0269	1.29	0.20	2.45	GTP-binding protein (hflX)
SSO0276	1.46	0.24	2.75	Small nuclear ribonucleoprotein (snRNP) homolog
SSO0931	1.42	0.12	2.67	Predicted transcriptional regulator
SSO0944	1.04	0.07	2.05	Hypothetical protein
SSO0980	1.30	0.05	2.47	Transposase ISC1217
SSO0999	1.58	0.22	2.99	ABC transporter
SSO1000	1.09	0.21	2.13	Maltose transport inner membrane protein
SSO1004	1.42	0.21	2.67	FAD linked oxidase homologue
SSO10449	1.03	0.07	2.04	Predicted transcriptional regulator
SSO10452	1.24	0.09	2.36	Hypothetical protein
SSO11076	1.44	0.12	2.71	Glycosyltransferase, putative
SSO1125	1.05	0.21	2.08	DsrE/DsrF-like family homologue
SSO1141	1.43	0.36	2.69	Copper binding protein
SSO1152	1.05	0.09	2.07	Peptidase U62 modulator of DNA gyrase homolog
SSO11550	1.27	0.19	2.42	STIV infection differentially regulated gene
SSO11575	1.41	0.19	2.65	Hypothetical protein
SSO1162	1.52	0.13	2.86	Multidrug resistance protein
SSO1268	1.05	0.21	2.07	Glutamine-fructose-6-phosphate transaminase (isomerizing glmS-2)
SSO1281	1.15	0.27	2.21	Last part of transposase in ISC1250
SSO1282	1.93	0.31	3.81	Oligo/dipeptide transport, ATP binding protein . (dppD-2)
SSO1284	2.36	0.28	5.14	Oligo/dipeptide transport, permease protein (dppB-2)
SSO1312	1.75	0.23	3.36	Ring oxydation complex/ phenylacetic acid degradation related protein
SSO1334	1.63	0.29	3.10	Malate synthase, putative (aceB/mas)
SSO1389	1.61	0.34	3.05	csx1(CRISPR/cas system-associated protein)
SSO1439	0.80	0.06	1.74	cas'' (Nuclease subunits of Cas3)
SSO1424	1.45	0.24	2.74	csa5(CRISPR/cas system-associated protein so-called) 'small' subunit
SSO1425	1.21	0.22	2.32	csm3 (CRISPR-associated RAMP)
SSO1443	2.56	0.15	5.91	csa5 (CRISPR/cas system-associated protein)
SSO1691	1.16	0.12	2.23	Hypothetical protein

SSO1717	1.42	0.08	2.68	Second ORF in transposon ISC1048
SSO1812	1.85	0.27	3.60	SirA family protein homologue
SSO1814	1.16	0.07	2.23	Transposase ISC1234
SSO1842	1.35	0.49	2.55	Glyceraldehyde-3-phosphate dehydrogenase, NADP dependent (gapN-2)
SSO1870	1.85	0.11	3.60	Rusticyanin homologue
SSO1911	0.99	0.06	1.98	Hemerythrin
SSO1997	2.13	0.47	4.37	CRISPR-associated autoregulator, DevR family homolog
SSO2048	1.97	0.19	3.93	Hypothetical protein
SSO2059	1.25	0.38	2.38	Acetyl-CoA synthetase (acetate-CoA ligase acsA-6)
SSO2067	1.25	0.48	2.38	Indolepyruvate ferredoxin oxidoreductase alpha subunit (iorA)
SSO2131	1.30	0.34	2.46	Transcriptional regulator homolog (Lrp/AsnC family)
SSO2291	3.00	0.00	7.98	Protein kinase, putative
SSO2617	1.04	0.11	2.06	Dipeptide ABC transporter permease protein dppC-3
SSO2656	0.99	0.04	1.99	Quinol oxidase (SoxABC), cytochrome B subunit (soxC)
SSO2657	1.41	0.10	2.66	Quinol oxidase (SoxABC), cytochrome aa3 subunit (soxB)
SSO2660	1.23	0.09	2.34	Rieske iron-sulfur protein-1 (soxL-1)
SSO2661	1.22	0.08	2.33	Hypothetical protein
SSO2681	1.00	0.07	2.01	Hypothetical protein
SSO2690	2.11	0.15	4.30	Sulfolobus mercury resistance protein, MerI (Sulfolobusislandicus)
SSO2757	2.60	0.31	6.06	Pyruvic-ferredoxin oxidoreductase alpha chain (porA-2)
SSO2758	1.22	0.16	2.33	Pyruvic-ferredoxin oxidoreductase gamma chain (porG-2)
SSO2761	1.07	0.15	2.09	Acyl-CoA dehydrogenase (acd-5)
SSO2827	1.11	0.07	2.15	Predicted transcriptional regulator
SSO2831	1.06	0.03	2.08	AAA family ATPase
SSO2846	1.21	0.09	2.32	Extracellular ligand-binding receptor homolog
SSO2881	1.09	0.23	2.14	Fe-S oxidoreductase homologue
SSO2882	1.41	0.26	2.65	Transposase ISC1234
SSO2986	1.12	0.02	2.18	Predicted transcriptional regulator
SSO2991	2.00	0.18	4.01	Hypothetical protein
SSO3045	1.23	0.20	2.34	ABC transporter, ATP binding protein
SSO3054	1.76	0.40	3.38	Terminal oxidase, small hydrophobic subunit (doxE)
SSO1443	2.56	0.15	5.91	csa5 (CRISPR/cas system-associated protein)
SSO1691	1.16	0.12	2.23	Hypothetical protein
SSO1717	1.42	0.08	2.68	Second ORF in transposon ISC1048
SSO1812	1.85	0.27	3.60	SirA family protein homologue
SSO1814	1.16	0.07	2.23	Transposase ISC1234

SSO1842	1.35	0.49	2.55	Glyceraldehyde-3-phosphatedehydrogenase, NADP dependent (gapN-2)
SSO1870	1.85	0.11	3.60	Rusticyanin homologue
SSO1911	0.99	0.06	1.98	Hemerythrin
SSO1997	2.13	0.47	4.37	CRISPR-associated autoregulator, DevR family homolog
SSO2048	1.97	0.19	3.93	Hypothetical protein
SSO2059	1.25	0.38	2.38	Acetyl-CoA synthetase (acetate-CoA ligase acsA-6)
SSO2067	1.25	0.48	2.38	Indolepyruvate ferredoxin oxidoreductase alpha subunit (iorA)
SSO2131	1.30	0.34	2.46	Transcriptional regulator homolog (Lrp/AsnC family)
SSO2291	3.00	0.00	7.98	Protein kinase, putative
SSO2617	1.04	0.11	2.06	Dipeptide ABC transporter permease protein dppC-3
SSO2656	0.99	0.04	1.99	Quinol oxidase (SoxABC), cytochrome B subunit (soxC)
SSO2657	1.41	0.10	2.66	Quinol oxidase (SoxABC), cytochrome aa3 subunit (soxB)
SSO2660	1.23	0.09	2.34	Rieske iron-sulfur protein-1 (soxL-1)
SSO2661	1.22	0.08	2.33	Hypothetical protein
SSO2681	1.00	0.07	2.01	Hypothetical protein
SSO2690	2.11	0.15	4.30	Sulfolobus mercury resistance protein, MerI (Sulfolobusislandicus)
SSO2757	2.60	0.31	6.06	Pyruvic-ferredoxin oxidoreductase alpha chain (porA-2)
SSO2758	1.22	0.16	2.33	Pyruvic-ferredoxin oxidoreductase gamma chain (porG-2)
SSO2761	1.07	0.15	2.09	Acyl-CoA dehydrogenase (acd-5)
SSO2827	1.11	0.07	2.15	Predicted transcriptional regulator
SSO2831	1.06	0.03	2.08	AAA family ATPase
SSO2846	1.21	0.09	2.32	Extracellular ligand-binding receptor homolog
SSO2881	1.09	0.23	2.14	Fe-S oxidoreductase homologue
SSO2882	1.41	0.26	2.65	Transposase ISC1234
SSO2986	1.12	0.02	2.18	Predicted transcriptional regulator
SSO2991	2.00	0.18	4.01	Hypothetical protein
SSO3045	1.23	0.20	2.34	ABC transporter, ATP binding protein
SSO3123	1.38	0.17	2.60	Hypothetical protein
SSO3130	1.15	0.12	2.21	Iron-sulfur protein, conserved putative
SSO3131	1.19	0.12	2.28	Heterodisulfide reductase related protein
SSO3174	1.15	0.13	2.23	Histidine kinase (Sulfolobussolfataricus)
SSO3178	1.32	0.04	2.50	Hypothetical protein
SSO3188	1.64	0.11	3.12	Hypothetical protein
SSO5027	1.08	0.12	2.11	Hypothetical protein
SSO5098	1.42	0.15	2.67	Terminal oxidase, small hydrophobic subunit (doxE)
SSO7239	1.13	0.14	2.18	Conserved hypothetical protein

SSO9088	1.64	0.19	3.11	Intergenic region
SSO9180	1.01	0.02	2.02	7 KD DNA-binding protein
SSO9500	1.37	0.14	2.58	Conserved hypothetical protein
SSOLCTR1a_6470_1708-1767	1.07	0.00	2.11	CRISPR Locus A
SSOLCTR1a_6470_3373-3432	0.03	0.03	1.02	CRISPR Locus A
SSOLCTR1a_6470_6329-6394	0.68	0.02	1.60	CRISPR Locus A
SSOLCTR1b_5964_278-343	0.77	0.05	1.70	CRISPR Locus B
SSOLCTR1b_5964_1493-1557	0.63	0.01	1.55	CRISPR Locus B
SSOLCTR1b_5964	0.52	0.04	1.44	CRISPR Locus B
SSOLCTR1c_1993_10-71	1.41	0.08	2.66	CRISPR Locus C
SSOLCTR1c_1993	1.15	0.18	2.22	CRISPR Locus C
SSOLCTR1c_1993_1916-1980	0.94	0.01	1.91	CRISPR Locus C
SSOLCTR1d_6005_483-551	1.40	0.08	2.65	CRISPR Locus D
SSOLCTR1d_6005	0.93	0.17	1.91	CRISPR Locus D
SSOLCTR1d_6005_105-174	0.85	0.16	1.80	CRISPR Locus D
SSOLCTR1d_6005_5568-5630	1.27	0.02	2.42	CRISPR Locus D
SSOLCTR1e_402	0.03	0.05	1.02	CRISPR Locus E
SSOLCTR1f_5776_3689-3753	0.68	0.09	1.60	CRISPR Locus F
SSOLCTR1f_5776_4021-4090	0.00	0.04	1.00	CRISPR Locus F
SSOLCTR1f_5776_4350-4419	0.62	0.19	1.54	CRISPR Locus F
SSO0067	-1.65	0.15	-3.13	SSU ribosomal protein S2AB (rps2AB)
SSO0098	-1.19	0.16	-2.28	Methionine aminopeptidase 2
SSO0099	-1.70	0.06	-3.24	Conserved hypothetical protein
SSO0173	-1.24	0.01	-2.36	Aspartyl-tRNAsynthetase (aspS)
SSO0192	-1.64	0.04	-3.12	Glutaredoxin related protein
SSO0199	-2.21	0.07	-4.64	S-adenosylmethioninesynthetase (maT)
SSO0202	-1.31	0.23	-2.48	D-arabino 3-hexulose 6-phosphate formaldehyde lyase (hpS-2)
SSO0217	-1.19	0.26	-2.28	SSU ribosomal protein S7AB (rpS7AB)
SSO0230	-1.49	0.18	-2.81	Nucleoside diphosphate kinase (NDP kinase) (ndk)
SSO0235	-1.05	0.02	-2.08	Radical SAM protein homolog
SSO0240	-1.26	0.22	-2.39	Adenylosuccinatelyase (adenylosuccinase)(ASL) (purB)
SSO0256	-1.11	0.02	-2.16	Conserved hypothetical protein
SSO0292	-1.32	0.10	-2.50	exosome complex RNA-binding protein Csl4 homolog
SSO0320	-1.13	0.20	-2.19	Hypothetical protein
SSO0352	-1.14	0.05	-2.20	Apoptosis-related Tfar19 related protein

SSO0353	-1.39	0.14	-2.63	SSU ribosomal protein S19E (rps19E)
SSO0356	-1.67	0.00	-3.17	Phosphate regulatory protein, putative
SSO0358	-1.11	0.27	-2.16	Conserved hypothetical protein
SSO0363	-1.06	0.16	-2.09	Prolidase
SSO0397	-1.60	0.15	-3.04	Proliferating cell nuclear antigen putative homolog
SSO0408	-1.48	0.09	-2.79	SSU ribosomal protein S13E
SSO0415	-1.54	0.18	-2.91	DNA-directed RNA polymerase, subunit E (rpoE1)
SSO0420	-1.16	0.04	-2.24	Reverse gyrase (topR-1)
SSO0435	-1.71	0.03	-3.28	SSU ribosomal protein S24E (rps24E)
SSO0436	-1.30	0.28	-2.46	Thiazole biosynthetic enzyme
SSO0439	-1.08	0.20	-2.11	tRNA intron endonuclease, putative
SSO0460	-1.62	0.03	-3.08	MRP protein homolog, conserved ATPase (mrp)
SSO0481	-1.16	0.26	-2.23	HAD-superfamily hydrolase homolog
SSO0501	-1.10	0.06	-2.15	Conserved hypothetical protein
SSO0503	-1.23	0.04	-2.35	Conserved hypothetical protein
SSO0553	-1.14	0.17	-2.20	Conserved hypothetical protein
SSO0554	-1.30	0.37	-2.46	Ribosomal protein L11 methyltransferase, putative
SSO0555	-1.31	0.10	-2.48	Conserved hypothetical protein
SSO0608	-1.42	0.37	-2.67	Conserved hypothetical protein
SSO0098	-1.19	0.16	-2.28	Methionine aminopeptidase 2
SSO0099	-1.70	0.06	-3.24	Conserved hypothetical protein
SSO0173	-1.24	0.01	-2.36	Aspartyl-tRNAsynthetase (aspS)
SSO0192	-1.64	0.04	-3.12	Glutaredoxin related protein
SSO0199	-2.21	0.07	-4.64	S-adenosylmethioninesynthetase (maT)
SSO0202	-1.31	0.23	-2.48	D-arabino 3-hexulose 6-phosphate formaldehyde lyase (hpS-2)
SSO0217	-1.19	0.26	-2.28	SSU ribosomal protein S7AB (rpS7AB)
SSO0230	-1.49	0.18	-2.81	Nucleoside diphosphate kinase (NDP kinase) (ndk)
SSO0235	-1.05	0.02	-2.08	Radical SAM protein homolog
SSO0240	-1.26	0.22	-2.39	Adenylosuccinatelyase (adenylosuccinase)(ASL) (purB)
SSO0256	-1.11	0.02	-2.16	Conserved hypothetical protein
SSO0292	-1.32	0.10	-2.50	exosome complex RNA-binding protein Csl4 homolog
SSO0320	-1.13	0.20	-2.19	Hypothetical protein
SSO0352	-1.14	0.05	-2.20	Apoptosis-related Tfar19 related protein
SSO0353	-1.39	0.14	-2.63	SSU ribosomal protein S19E (rps19E)
SSO0356	-1.67	0.00	-3.17	Phosphate regulatory protein, putative
SSO0358	-1.11	0.27	-2.16	Conserved hypothetical protein
SSO0363	-1.06	0.16	-2.09	Prolidase

SSO0397	-1.60	0.15	-3.04	Proliferating cell nuclear antigen putative homolog
SSO0408	-1.48	0.09	-2.79	SSU ribosomal protein S13E
SSO0415	-1.54	0.18	-2.91	DNA-directed RNA polymerase, subunit E (rpoE1)
SSO0420	-1.16	0.04	-2.24	Reverse gyrase (topR-1)
SSO0435	-1.71	0.03	-3.28	SSU ribosomal protein S24E (rps24E)
SSO0436	-1.30	0.28	-2.46	Thiazole biosynthetic enzyme
SSO0439	-1.08	0.20	-2.11	tRNA intron endonuclease, putative
SSO0460	-1.62	0.03	-3.08	MRP protein homolog, conserved ATPase (mrp)
SSO0481	-1.16	0.26	-2.23	HAD-superfamily hydrolase homolog
SSO0501	-1.10	0.06	-2.15	Conserved hypothetical protein
SSO0503	-1.23	0.04	-2.35	Conserved hypothetical protein
SSO0553	-1.14	0.17	-2.20	Conserved hypothetical protein
SSO0554	-1.30	0.37	-2.46	Ribosomal protein L11 methyltransferase, putative
SSO0555	-1.31	0.10	-2.48	Conserved hypothetical protein
SSO0608	-1.42	0.37	-2.67	Conserved hypothetical protein
SSO0626	-1.96	0.30	-3.88	Phosphoribosylaminoimidazole-succinocarboxamide synthase (SAICAR synthetase) (purC)
SSO0708	-1.07	0.15	-2.09	LSU ribosomal protein L14AB (rpl14AB)
SSO0757	-1.16	0.06	-2.23	Spermidine synthase
SSO0771	-1.22	0.21	-2.34	Cell division control 6/orc1 protein homolog (cdc6-2)
SSO0881	-1.47	0.01	-2.78	VPS24 Conserved protein implicated in secretion homolog
SSO0886	-1.20	0.12	-2.30	myo-inositol-1-phosphate synthase
SSO0911	-1.19	0.01	-2.28	Cell division protein
SSO0946	-1.89	0.09	-3.70	Transcription initiation factor IIB (TFIIB) homolog (TFB-2)
SSO0951	-1.37	0.19	-2.58	TATA box binding protein, hypothetical (tfIID)
SSO0981	-1.21	0.07	-2.31	Pyruvate kinase (pyK)
SSO10285	-1.35	0.15	-2.55	Coenzyme PQQ synthesis protein
SSO12199	-1.08	0.06	-2.11	Nucleotide pyrophosphohydrolase
SSO1889	-1.66	0.24	-3.16	ATP-dependent RNA helicase
SSO1890	-1.15	0.10	-2.23	major facilitator superfamily MFS_1 homolog
SSO2089	-1.07	0.14	-2.09	TenA family transcriptional regulator homolog
SSO2146	-1.28	0.03	-2.42	major facilitator superfamily MFS_1 homolog
SSO2182	-1.13	0.14	-2.19	Isocitrate dehydrogenase
SSO2190	-1.19	0.10	-2.29	Hypothetical protein
SSO2222	-1.08	0.23	-2.11	Thioredoxin reductase (trxB-1)
SSO2231	-1.59	0.33	-3.00	Hypothetical protein
SSO2255	-1.15	0.05	-2.22	Peroxiredoxin, bacterioferritincomigratory protein homolog (bcp-3)

SSO2279	-1.69	0.06	-3.23	Hypothetical protein
SSO2292	-2.11	0.31	-4.31	Amino acid transporter related protein
SSO2390	-1.57	0.12	-2.98	Inorganic pyrophosphatase, putative (ppa)
SSO2407	-1.36	0.02	-2.56	2-isopropylmalate synthase, putative (leuA-3)
SSO2423	-1.01	0.05	-2.02	Type I phosphodiesterase/nucleotide pyrophosphatase homolog
SSO2431	-1.23	0.20	-2.35	Carbon monoxide dehydrogenase subunit G homolog
SSO2433	-1.09	0.16	-2.12	Carbon monoxide dehydrogenase, small chain (cutC-1)
SSO2583	-1.22	0.04	-2.33	Sulfolipid biosynthesis protein (sqdB)
SSO2585	-1.18	0.26	-2.27	L-lactate dehydrogenase
SSO2635	-1.12	0.03	-2.17	Hypothetical protein
SSO2653	-1.48	0.08	-2.79	Conserved hypothetical protein
SSO2706	-1.03	0.11	-2.04	5-methylthioadenosine phosphorylase (mtaP)
SSO2778	-1.08	0.06	-2.11	UspA domain protein homolog
SSO3003	-1.14	0.06	-2.21	Glucose 1-dehydrogenase (dhg-1)
SSO3189	-2.58	0.02	-6.00	Amino acid transporter related protein
SSO3200	-1.11	0.07	-2.15	Conserved hypothetical protein
SSO3219	-1.04	0.18	-2.06	Sugar phosphate nucleotydyltransferase
SSO3224	-1.32	0.07	-2.50	Amino acid transporter related protein
SSO3226	-1.06	0.17	-2.09	Fructose-bisphosphate aldolase homolog
SSO5345	-0.97	0.06	-1.96	Traslation elongation factor EF-1 beta subunit
SSO5478	-1.54	0.18	-2.91	LSU ribosomal protein L24E (rpl24E)
SSO5544	-1.20	0.01	-2.30	Carboxylate-amine ligase
SSO5668	-0.96	0.07	-1.94	LSU ribosomal protein LX
SSO5798	-1.16	0.09	-2.24	DNA-directed RNA polymerase, subunit E (rpoE2)
SSO6264	-1.57	0.26	-2.98	Conserved hypothetical protein
SSO6453	-1.20	0.04	-2.29	LSU ribosomal protein L37E (rpl37E)
SSO6830	-1.23	0.18	-2.35	STIV infection differentially regulated gene
SSV2_A305	-1.54	0.04	-2.91	SSV2 ORF

Table S5. *S. solfataricus* SSV1/SSV2-InF1 infected strain: a complete list of the up-regulated and down-regulated genes throughout the growth.

Similarly regulated in the SSV1-InF1 infected strain
Similarly regulated in the SSV2-InF1 infected strain
Similarly regulated in both single-infected strains

Gene ID	Average log2 Ratio	S.D. log2 Ratio	VGEF	Function
non-coding-RNA_Sso-181	1.07	0.06	2.11	non-coding RNA
SSO0276	1.67	0.06	3.18	Like-Sm ribonucleoprotein core homologue
SSO0445	1.14	0.15	2.20	Agmatinase (agmatineureohydrolase) (speB-1)
SSO0535	1.04	0.06	2.06	Acyl carrier protein synthase
SSO0544	1.02	0.05	2.03	ExsB family protein homologue
SSO1052	1.16	0.08	2.23	Oligosaccharyltransferase STT3 subunit (B5)
SSO1284	1.07	0.07	2.10	Oligo/dipeptide transport, permease protein (dppB-2)
SSO1312	1.46	0.27	2.74	Ring oxidation complex/ phenylacetic acid degradation related protein
SSO1859	1.11	0.04	2.16	Heat shock protein (htpX-1)
SSO2048	1.28	0.12	2.44	Hypothetical protein
SSO3051	1.41	0.10	2.66	Alpha-glucosidase (malA)
SSO0099	-1.22	0.15	-2.34	Glutaredoxin related protein, metal-dependent hydrolase
SSO0192	-1.15	0.11	-2.22	Glutaredoxin related protein
SSO0356	-1.37	0.33	-2.58	Phosphate regulatory protein, putative
SSO0397	-1.09	0.10	-2.13	Proliferating cell nuclear antigen putative homologue
SSO0420	-1.07	0.04	-2.10	Reverse gyrase (topR-1)
SSO0437	-1.16	0.17	-2.23	3-octaprenyl-4-hydroxybenzoate decarboxylase
SSO0460	-1.14	0.16	-2.20	MRP protein homologue, conserved ATPase (mrp)
SSO0503	-1.11	0.15	-2.16	Hypothetical protein
SSO0626	-1.34	0.25	-2.53	Phosphoribosylaminoimidazole-succinocarboxamide synthase
SSO0881	-1.19	0.08	-2.28	Hypothetical protein
SSO0951	-1.26	0.11	-2.40	TATA box binding protein, hypothetical (tfIID)
SSO1877	-1.25	0.06	-2.37	Small metal-binding protein
SSO1889	-1.03	0.35	-2.05	ATP-dependent RNA helicase
SSO2292	-1.89	0.30	-3.71	Amino acid transporter, putative
SSO2390	-1.01	0.03	-2.02	Inorganic pyrophosphatase, putative (ppa)
SSO3189	-1.97	0.21	-3.93	Amino acid transporter, putative
SSO5544	-1.23	0.21	-2.35	Carboxylate-ammine ligase
SSO5847	-1.01	0.26	-2.01	Hypothetical protein
SSV2_A305	-1.20	0.26	-2.29	SSV2 ORF

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