DEVELOPMENT OF MICROBIAL AND/OR ENZYMATIC SYSTEMS FOR THE VALORIZATION OF LIGNOCELLULOSIC WASTES

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"The fuel of the future is going to come from apples, grass, sawdust. There is fuel in every bit of vegetable matter that be fermented."

Henry Ford,1925

Alla mia famiglia e

alle persone che mi hanno accompagnato in questo percorso

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SUMMARY

The future trend of white biotechnology is the seek of renewable resources for the production of goods, traditionally obtained from petrol. Shifting society's dependence away from petroleum to renewable biomass resources is generally viewed as an important contribute to the development of a sustainable industrial society and the reduction of greenhouse gas emissions. Among the available resources that are exploitable for biofuel production, there are the residues coming from different human activities such as agriculture (i.e.: cereals straws), food processing-industry, forestry, green and organic fractions of urban wastes. Their exploitation can contribute to the reduction of the price of the bioethanol production process, raw materials being responsible of almost half of the total production cost. A further element contributing to soaring biofuels costs, is constituted by enzymes. Their use roughly doubles the cost of cellulosic ethanol production and lessens the economic advantages of using waste materials. A valide alternative to purified/commercial enzymes may be the direct production of the enzymes of interest on the lignocellulosic material to be converted through its microbial fermentation. At this purpose, a powerful technique is solid state fermentation (SSF). This fermentative technique reproduces conditions really close to the natural environment in which many higher filamentous fungi have evolved. These microorganisms can be exploited both for wastes pretreatment, for the removal of lignin and hemicellulose hydrolysis, and for the production of industrially relevant enzymes (oxidative as well as hydolytic activities) for further applications. As a matter of fact, lignocellulosic wastes may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis ensuring efficient production of lignino-cellulolytic enzymes.

In the present study the residues from tomato and apple processing were chosen as raw materials. On the other side, as conversion microorganisms, the two white rot fungi *Pleurotus ostreatus* and *Trametes versicolor*, were selected. Culture conditions were set up, allowing waste colonization and transformation. This study showed the good potential of tomato pomace as substrate for laccases production by *P. ostreatus and T. versicolor* SSF, considering that significant enzyme activity levels were achieved without any optimization of culture conditions, neither by nutrient addition nor by O_2 enrichment. Furthermore, SSFs on tomato pomace hold enormous potential for protease production, giving activity levels higher than those reported for fungi typically considered as the best protease producers. A process of fungal SSF was developed on apple waste, identifying the parameters allowing fast substrate colonization by both fungi. It was shown that apple pomace induced high levels of xylanases, with *P. ostreatus* secreting higher levels than *T. versicolor*. Both *P. ostreatus* and *T. versicolor* secreted levels of laccase activities that are lower or comparable with those obtained on tomato pomace. *T. versicolor* was shown to produce Manganese peroxidase, even if at low levels. On this substrate, low levels of protease activity were obtained, for both microorganisms. Furthermore, both tomato and apple pomace SSFs were shown to be better systems than liquid culture for the production of high laccases levels by *P. ostreatus*. Moreover, as one of the most significant results of this study, the developed *P. ostreatus* SSF processes provide the production of two laccase isoforms not detected in any other liquid culture conditions analysed so far. Finally, a strategy for *P. ostreatus* xylanase enrichment was investigated. The first trials for the identification of xylanolytic enzymes allowed the identification of an α-galactosidase. This enzyme even though not involved in xylan main chain break-down, plays its role in the removal of galactose units from both galactomannans and arabinoxylans, acting as ancillar xylanolytic enzyme.

RIASSUNTO

1. Introduzione

Lo sviluppo di un'industria sostenibile da un punto di vista tanto ambientale quanto economico si realizza non solo attraverso il miglioramento dei processi di produzione tradizionali, ma anche tramite lo sviluppo di nuovi processi e prodotti a partire da fonti rinnovabili. La lignocellulosa rappresenta una componente abbondante degli scarti dei settori agro-alimentare ed agricolo, nonché dei rifiuti solidi urbani (frazione organica dei rifiuti e sfalci di potatura del verde urbano). La valorizzazione di materiali altrimenti destinati all'incenerimento o al confinamento in discarica si può realizzare attraverso lo sviluppo di un processo produttivo che impieghi gli scarti ligno-cellulosici, sia come materiali da cui estrarre composti d'interesse sia come substrati per processi di fermentazione microbica su stato solido (*upgrading concept*). Per fermentazione su stato solido si intende un qualsiasi processo che si verifichi nella assenza totale o parziale di acqua libera, e che impieghi un substrato naturale come fonte di nutrienti e supporto solido per la crescita di microrganismi selezionati, o un supporto inerte impregnato con un appropriato mezzo di coltura.

L'elevato contenuto in polisaccaridi complessi (cellulosa, emicellulosa, pectina) e in zuccheri facilmente assimilabili da microrganismi rende gli scarti lignocellulosici adatti come substrati in processi in cui vengano utilizzati funghi come sistema biologico per l'ottenimento di composti di rilevanza industriale. Infatti, i funghi filamentosi sono considerati biocatalizzatori d'eccellenza in quanto la fermentazione su stato solido (SSF) simula le condizioni naturali in cui essi si sono evoluti in natura, producendo diverse attività enzimatiche coinvolte nella trasformazione della lignocellulosa. La lignocellulosa rappresenta una promettente risorsa rinnovabile per la produzione di bioetanolo attraverso un processo che convenzionalmente prevede una fase di pretrattamento chimico-fisico per rimuovere la lignina e incrementare l'accessibilità dei polisaccaridi per la successiva fase di idrolisi. Tali metodi hanno lo svantaggio di utilizzare grandi quantità di solventi chimici e produrre reflui inquinanti, o richiedere un elevato input energetico. D'altra parte, l'idrolisi dei polisaccaridi viene realizzata grazie all'impiego di acidi forti o di cellulasi commercialmente disponibili, con il conseguente incremento dei costi di produzione dei biocombustibili. In alternativa, è possibile sfruttare la capacità dei funghi lignino-cellulolitici di colonizzare la lignocellulosa, grazie alla produzione di ligninasi e glicosil idrolisi. La fermentazione su stato solido degli scarti lignocellulosici consentirebbe, inoltre, di ottenere la produzione degli enzimi necessari per l'idrolisi di cellulosa ed emicellulosa, direttamente sul materiale da trattare, evitando il ricorso ad enzimi purificati. In tal modo, quindi, si potrebbero ridurre gli svantaggi tanto economici -costi dei reagenti, della strumentazione, del processo di trattamento delle acque- quanto ecologici, quali la produzione di grandi volumi di acque reflue, dovuti all'impiego di composti chimici.

Oltre agli enzimi ossidativi e idrolitici, un ulteriore prodotto d'interesse ottenibile tramite SSF degli scarti lignocellulosici con i funghi selezionati è rappresentato dai monosaccaridi, derivanti dall'idrolisi della componente polisaccaridica. Tali zuccheri possono essere convertiti in acidi per la produzione di materiali polimerici o in bio-combustibili, quali bio-etanolo e butanolo. Inoltre, caratteristiche peculiari della SSF quali la ridotta richiesta energetica, ridotti volumi dei fermentatori e minori rischi di contaminazione, assenza di macchinari complessi e di complessi sistemi di controllo, rendono la produzione di composti ad alto valore aggiunto maggiormente conveniente. La concomitante commercializzazione dei diversi prodotti (biocombustibili, enzimi, molecole d'interesse industriale) concorrerebbe a

compensare la ridotta competitività economica dei biocombustibili rispetto a quelli di origine fossile. In tal modo, quindi, si otterrebbe l'integrazione di diverse linee produttive al fine di incrementare la redditività economica del processo, e ridurre parallelamente il volume del flusso di scarti in uscita dall'impianto, realizzando una vera e propria bioraffineria. Ad esempio, il recupero di molecole d'elevato valore commerciale e la produzione di enzimi d'interesse industriale concorrerebbero ad incrementare la convenienza del processo di produzione del bio-etanolo.
In tale ambito si inserisce il progetto di dottorato descritto che ha l'obiettivo di sviluppare biosistemi microbici e/o enzimatici per la valorizzazione di materiali lignocellulosici di scarto, grazie alla produzione di composti d'interesse commerciale. A tale scopo il lavoro è stato articolato nelle seguenti fasi:

1. Selezione di materiali lignocellulosici di scarto
2. Selezione di funghi lignino-cellulolitici
3. Messa a punto di processi di fermentazione su stato solido delle matrici lignocellulosiche selezionate
4. Individuazione delle condizioni per la produzione di composti ad alto valore aggiunto.

2. Risultati

2.1 Selezione dei materiali lignocellulosici di scarto

Il primo obiettivo del progetto di dottorato è stata la selezione di materiali lignocellulosici di scarto prodotti sia sul territorio nazionale che su quello Campano. I parametri tenuti in considerazione per la selezione degli scarti sono stati i volumi prodotti, la stagionalità di produzione, l'esistenza di ulteriori processi per il loro riutilizzo e la composizione macromolecolare (contenuto di cellulosa e emicellulosa). La categoria presa in esame è quella dei rifiuti speciali non pericolosi, di cui fanno parte i residui agricoli, quelli agro-industriali, gli sfalci di potatura e la frazione organica dei rifiuti solidi urbani. I dati sono stati raccolti a partire da fonti quali i rapporti ufficiali dell'Agenzia per la Protezione dell'Ambiente e del Territorio -APAT- stilato congiuntamente all'Osservatorio Nazionale sui Rifiuti -ONR- e dell'ISTAT, e da interviste dirette o sono di natura bibliografica. L'analisi ha evidenziato come potenzialmente interessanti i residui agricoli derivanti da grano duro e tenero, pomodori, vite, clementine, olive, arance, in Italia; quelli derivanti da patate, pomodori, grano duro, pesche e nocciole in Campania e come scarti delle lavorazioni agro-industriali quelli prodotti dalla trasformazione di pomodoro, uva, e mele.
L'impiego dei residui agricoli è stato, però, escluso nella messa a punto del processo di fermentazione su stato solido, dal momento che essi sono solitamente lasciati sul campo, avendo un importante ruolo nei processi di umificazione responsabili del mantenimento della fertilità dei terreni ed inoltre la loro raccolta implicherebbe un costo non trascurabile. Gli scarti agro-industriali sono stati selezionati in quanto è stata dimostrata la necessità da parte delle industrie di trasformazione, presenti sul territorio, di smaltire i grandi volumi prodotti. Inoltre, per lo sviluppo di un processo microbico e/o enzimatico è interessante il fatto che ogni scarto è caratterizzato da una composizione macromolecolare omogenea. Gli scarti della lavorazione del pomodoro sono stati scelti dal momento che sono prodotti in volumi cospicui sia sul territorio nazionale che su quello Campano. D'altra parte, gli scarti della lavorazione della mela, seppure prodotti in minore quantità, sono risultati interessanti soprattutto per la presenza di molecole con un elevato potenziale di mercato. Inoltre, gli scarti di mela e pomodoro sono prodotti in due intervalli temporali contigui (Luglio-Settembre e Ottobre-Dicembre, rispettivamente), consentendo un più duraturo rifornimento di

materiale all'impianto di trasformazione. Come ulteriore substrato d'interesse per lo sviluppo del processo, la frazione organica dei rifiuti presenta il vantaggio di essere prodotta senza soluzione di continuità, in volumi significativi anche se con una composizione merceologica variabile nel corso dell'anno.

2.2 Selezione di funghi lignino-cellulolitici

Un ulteriore obiettivo del progetto è stata la selezione di microrganismi in grado di convertire le diverse componenti macromolecolari presenti negli scarti quali lignina, pectina, emicellulosa e cellulosa. Si è scelto di impiegare i funghi come microrganismi per la conversione degli scarti, poiché essi sono in grado di colonizzare materiali lignocellulosici, le cui caratteristiche di umidità e struttura sono prossime a quelle proprie dell'ambente naturale dei funghi. L'analisi dei dati disponibili in letteratura è stata condotta valutando da un lato la variazione de contenuto delle singole componenti macromolecolari dovuta alla crescita dei funghi, dall'altro i livelli di produzione delle attività enzimatiche coinvolte nel processo di conversione da parte dei funghi. La valutazione dei dati bibliografici raccolti ha portato alla creazione di una classifica di funghi lignino-cellulolitici, in base alle *performances* di conversione delle diverse componenti macromolecolari e di produzione delle attività enzimatiche correlate. *Trametes versicolor* NBRC (IFO) 4937 è stato selezionato come fungo lignino-cellulolitico dal momento che è in grado di trasformare con pari efficienza sia lignina che cellulosa. I ceppi PT1 and PT4 di *P. tuber-regium* possono essere classificati come convertitori di emicellulosa, dal momento che determinano una significativa riduzione del contenuto di emicellulosa piuttosto che delle altre frazioni. *Phellinus pini,* d'altro canto, risulta il migliore degradatore di lignina rispetto a tutti i ceppi fungini analizzati. Per quanto riguarda, invece, i livelli di attività enzimatiche secrete, *Moniliella* spSB9 produce elevati livelli di poligalatturonasi e di pectinasi, su diversi substrati. *P. ostreatus* 2191 risulta essere il migliore produttore di endoglucanasi, mentre *P. ostreatus* 2175 produce i livelli più elevati di xilanasi e di *Filter Paper activity* (attività endo- ed eso-glucanasica). *Aspergillus niger* BTL e *Neurospora crassa* DSM1129, d'altra parte, secernono quantitativi elevati di diverse attività enzmatiche quali poligalatturonasi, pectinasi, endoglucanasi e xilanasi. Allo stesso modo, *P. dryinus* IBB903, produce su diversi substrati elevati livelli di diverse glicosil idrolasi. Questi due funghi potrebbero essere impiegati per la contemporanea trasformazione delle diverse componenti polisaccaridiche. E' rilevante sottolineare che le *performances* dei microrganismi nel processo di conversione sono fortemente influenzate dal substrato di crescita. Risulta, quindi, particolarmente interessante la versatilità metabolica mostrata dai funghi appartenenti al genere *Pleurotus*.
Sulla base delle analisi dei dati bibliografici sono stati, quindi, selezionati i funghi *P. ostreatus* ATCC MYA-2306 (type: Florida) e *T. versicolor* NBRC(IFO) 4937, come microrganismi lignino-cellulolitici per lo sviluppo del processo di valorizzazione degli scarti di lavorazione di pomodoro e mela. Inoltre, il ceppo di *P. ostreatus* è da tempo oggetto di studio nel laboratorio in cui questo progetto di dottorato è stato realizzato, e ne è stata ampiamente caratterizzata una famiglia di cinque isoenzimi ligninolitici laccasi.

2.3 Messa a punto di un processo di fermentazione su stato solido delle matrici ligno-cellulosiche selezionate

Nell'ambito di uno stage svolto presso il laboratorio facente capo al Dr. Christopher Augur, dell'*Institut de recherche pour le développement* (IRD) dell'Università Paul

Cézanne di Marsiglia (Francia) sono state messe a punto le condizioni di fermentazione su piccola scala di un microrganismo modello su di un substrato modello da noi formulato, impiegando sia beute che un bio-reattore a colonne con flusso di aria umidificata e controllo della temperatura. Al fine di mettere a punto le condizioni sperimentali per l'allestimento delle fermentazioni su stato solido si è proceduto in primo luogo alla definizione di un substrato modello (denominato Modello verde) che avesse una composizione macromolecolare analoga a quella di uno scarto vegetale reale. Il modello verde è stato formulato unendo uguali quantitativi di otto differenti vegetali quali broccoli 'friarielli', lattuga, scarola, broccoli baresi, verza, spinaci, cicoria e broccoli neri, e ne è stata determinata la composizione macromolecolare (lignina: 2.6 % w/w, cellulosa 18.2 % w/w, emicellulosa: 4.2 % w/w). Come microrganismo modello è stato scelto il fungo ifomicete *Trichoderma harzianum* F470 (Roussos, 1987), di cui è nota la capacità di produrre e secernere in condizioni di SSF elevati titoli di cellulasi. Inoltre, all'interno dei laboratori dell'IRD è stata caratterizzata la fisiologia di crescita del fungo su bagassa di canna da zucchero impiegata sia come substrato che come supporto.
In prima battuta, sono state individuate le condizioni che consentono di ottenere un elevato numero di spore del microrganismo selezionato. Si è, in seguito, verificata la capacità del fungo di colonizzare il modello verde nelle condizioni applicate, utilizzando il bio-reattore a colonne brevettato da Raimbault (1989), con un flusso di aria umidificata. In tal modo, si è verificata la necessità di modificare sia tempo di rivoltamento che volume dell'inoculo, in quanto essi condizionano significativamente l'omogeneità del sistema.
Il processo fermentativo è stato poi trasferito in un sistema statico senza passaggio di aria, quale le beute, che rappresentano il sistema poi utilizzato con gli scarti reali, verificando che il fungo riesce a colonizzare il substrato, senza determinare significative variazioni nell'umidità del sistema. Sono state, inoltre, condotte prove impiegando come supporto non più la bagassa di canna da zucchero, ma gli sfalci di potatura degli ulivi, essendo questi ultimi di più facile reperimento in Italia così come in Campania. Tali esperimenti hanno consentito di definire l'influenza dei diversi parametri sull'allestimento di fermentazioni su stato solido (pH, umidità, aerazione) e le condizioni per la preparazione del substrato, quali le dimensioni delle particelle dello scarto (0.8 <x< 2 mm). Si è, inoltre, appurato che è necessario operare in condizioni sterili, avendo *T. harzianum* un micelio molto fragile. Una ulteriore indicazione ottenuta è che il pH deve essere modificato, portandolo a valori più simili a quelli ottimali per il fungo ifomicete (pH: 5.6).

2.4 Individuazione delle condizioni per la produzione di composti ad alto valore aggiunto.

2.4.1 Sviluppo di un processo di valorizzazione di scarti di lavorazione del pomodoro con i funghi *P. ostreatus* e *T. versicolor*

6 millioni di tonnellate di pomodori sono stati trasformati nel 2005, dando vita a circa 120,000 tonnellate di scarti. Nello stesso anno in Campania sono stati prodotti circa 10,000 tonnellate di scarti (*www.anicav.it*) a partire dalle 50,000 tonnellate di pomodori processate. Il materiale, raccolto presso un impianto di trasformazione situato in Campania, è stato impiegato come substrato per l'allestimento di esperimenti di fermentazione su stato solido con i due funghi *P. ostreatus* e *T. versicolor*. Al fine di ottenere la completa colonizzazione del substrato si è proceduto all'individuazione delle condizioni di umidità, pH, dimensioni delle particelle, aggiunta di un materiale come supporto, tasso di inoculazione ottimali.

Per quanto riguarda *P. ostreatus*, il processo sviluppato ha consentito di individuare le condizioni per l'ottenimento di livelli rilevanti di attività enzimatiche di rilievo industriale, quali proteasi (13.000 U/g sostanza secca, tra quarto e sesto giorno), laccasi (15 U/g s.s., tra terzo e quarto giorno), xilanasi (due picchi intorno a 10 U/g s.s., rispettivamente tra il primo ed il terzo giorno, e tra settimo e nono giorno). Per quanto riguarda l'attività laccasica si è riscontrata la produzione di due isoenzimi mai riscontrati nelle condizioni sperimentali adottate in precedenza. Inoltre, *P. ostreatus* secerne su pomodoro un'unica isoforma ad attività xilanasica al secondo giorno, e due isoenzimi con diversa mobilità elettroforetica al settimo giorno. Per *T. versicolor*, d'altro canto, i livelli sia di attività laccasica che xilanasica sono più alti che per *P. ostreatus*, anche se i tempi di produzione sono più lunghi. Il massimo di attività laccasica si ottiene al sedicesimo giorno 35 U/g s.s., mentre l'attività xilanasica subisce un costante incremento fino al massimo di circa 50 U/g s.s. (tredicesimo giorno). Per l'attività proteasica si ottengono valori analoghi a quelli ottenuti per *P. ostreatus*, ma su tempi più lunghi (tredicesimo giorno). Inoltre, per quanto riguarda sia laccasi che xilanasi si osserva la produzione di un'unica isoforma a differenza di quanto riscontrato per l'altro fungo preso in esame. Per entrambi i funghi non è stata riscontrata alcuna produzione di attività cellulasica.

2.4.2 Sviluppo di un processo di valorizzazione di scarti di lavorazione di mela con i funghi *P. ostreatus* e *T. versicolor*

Le mele costituiscono una delle principali colture in Italia, con 2 milioni di tonnellate prodotte nel 2006 su scala nazionale, portando l'Italia tra i principali paesi poduttori in Europa e al quinto posto su scala mondiale. Inoltre, la Campania spicca con circa 70.000 tonnellate di mele prodotte nel 2006 (Istat, Coltivazioni 2008). Il 5 % della frutta raccolta viene trasformata dando vita a circa 2.000 tonnellate in Italia e 70 tonnellate in Campania come volumi di scarti solidi prodotti. Questi scarti sono solitamente utilizzati come fertilizzanti o come mangimi animali. Le mele mostrano bassi livelli di lignina ed emicellulosa, ed un più alto contenuto di cellulosa.

Lo scarto di mela è stato prelevato in un'azienda di trasformazione Campana. La diversa natura del materiale ha richiesto un diverso trattamento al fine di realizzare le condizioni appropriate per la colonizzazione da parte dei due funghi. I funghi hanno colonizzato rapidamente e in maniera paragonabile lo scarto di mela sia in presenza che in assenza del supporto, e quindi la sua aggiunta si è rivelata superflua. *P. ostreatus* si è rivelato in grado di metabolizzare gli zuccheri presenti in questo materiale, determinandone una iniziale riduzione, seguita poi da un incremento intorno al quinto giorno. L'attività xilanasica è fortemente indotta dallo scarto di mela, raggiungendo il valore di circa 80 U/g s.s. intorno al quarto giorno. L'andamento dell'attività laccasica, d'altro canto, è analogo a quanto osservato sugli scarti di pomodoro, sia nei livelli che nei tempi di produzione. Anche sullo scarto di mela sono state evidenziate le isoforme ad attività laccasica indotte sullo scarto di lavorazione del pomodoro. I livelli d'attività proteasica, significativamente minori su mela piuttosto che su pomodoro, si possono ascrivere al più basso contenuto proteico del primo scarto rispetto al secondo. Per quanto concerne *T. versicolor*, i livelli di zuccheri riducenti si attestano per tutta la durata della fermentazione sui valori iniziali. La produzione sia di attività laccasica che xilanasica sembra anticipata rispetto alla fermentazione su pomodoro. Infatti, l'attività xilanasica raggiunge già a secondo giorno il valore di circa 40 U/g s.s. che resta pressochè costante per tutta la durata della fermentazione; l'attività laccasica, d'altra parte, si attesta su valori più bassi che sul pomodoro (4 U/g s.s.) già a partire dal secondo giorno. Anche in questo

caso si osserva la secrezione di un unico isoenzima ad attività laccasica. Così come sul pomodoro, per entrambi i funghi non è stata riscontrata produzione di attività cellulasica.

2.5 Identificazione di xilanasi da *P. ostreatus*

Tra i prodotti ad alto valore aggiunto ottenuti attraverso il processo di fermentazione su stato solido sviluppato, particolare attenzione è stata rivolta agli enzimi ad attività xilanasica. Tale interesse è motivato dalle diverse applicazioni che questi enzimi trovano a livello industriale, quali la decolorazione della polpa di cellulosa nella produzione della carta, la chiarificazione di vino e succhi di frutta, il miglioramento della qualità dei prodotti da forno e la produzione di mangime per animali (Berg ***et al***., 2001). Il settore della produzione della carta è quello che più si avvantaggia dell'uso di xilanasi in particolare nella fase di sbiancamento, dove il loro utilizzo consente di sostituire fino al 20-30% del cloro impiegato, riducendo i volumi di acque reflue tossiche da trattare.

La consultazione dei dati disponibili in letteratura ha evidenziato che non sono state finora caratterizzate xilanasi da *P. ostreatus*. D'altra parte, diversi geni codificanti enzimi ad attività xilanasica sono stati annotati nel genoma del fungo (*http://genome.jgi-psf.org/PleosPC15_1*). Si è perciò allestita una procedura di arricchimento di enzimi xilanolitici da *P. ostreatus*, che ha previsto una cromatografia a scambio idrofobico (Phenyl Sepharose) seguita da una a scambio anionico (DEAE Sepharose). Tale approccio ha consentito la separazione di due picchi cromatografici ad attività xilanasica. La frazione cromatografia con la maggiore attività enzimatica è stata sottoposta ad analisi di proteomica, volte all'identificazione della/e proteina/e ad attività xilanasica. I risultati finora ottenuti non hanno consentito l'identificazione di xilanasi. E' stato, tuttavia, possibile identificare una proteina coinvolta del metabolismo dei polisaccaridi, corrispondente ad una α-galattosidasi (De Vries ***et al.***, 1999). Tale enzima, come evinto da dati di letteratura, risulta coinvolto nell'idrolisi degli arabinoxilani (galattosio + xilosio/ arabinosio) e svolge una funzione ancillare nell'idrolisi dell'emicellulosa, liberando la catena principale dei galattomannani dal galattosio, presente come gruppo sostituente.

1. Introduction

1.1 Current energy situation and future trends

The future trend of white biotechnology is the seek of new renewable resources for the production of goods, traditionally obtained from petrol (figure 1.1). At the beginning of the 20th century, many industrial materials such as dyes, solvents, and synthetic fibres were made from natural sources, such as trees and agricultural crops. From the late 1960s on, petroleum derivatives started to displace the bio-based products; only the 1970s energy crisis arised new interest in the recruitment of new sources for fuels and materials. However, the later reduction in oil prices reinforced the exploitation of petroleum in every possible field of application, bringing to the present oil shortages. Furthermore, energy demand is projected to grow by more than 50% by 2025. Of course, finite oil resources cannot provide a safe perspective on a global scale for the long term. As pointed out by many researchers (Hoffert, 2002), future reductions in the ecological footprint of energy generation will reside in a multifaceted approach, involving hydrogen, primary energy sources such as solar and wind energy, and biofuels. Shifting society's dependence away from petroleum to renewable biomass resources is generally viewed as an important contribute to the development of a sustainable industrial society and the reduction of greenhouse gas emissions.

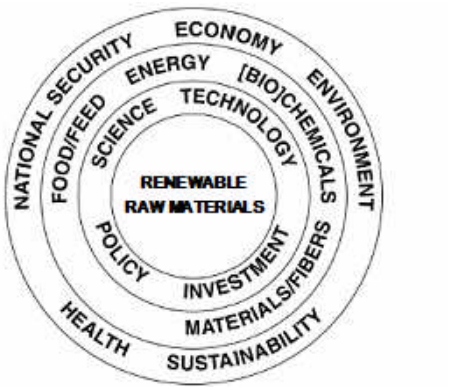

Figure 1.1: Renewable raw materials as central issue for alternative strategies of sustainable development

The U.S. Department of Energy has set the goals to replace 30% of the liquid petroleum transportation fuel with biofuels and 25% of industrial organic chemicals with biobased products by 2025. The European Union with the Biofuels Directive 2003/30/EC, adopted in 2003, settled that 2% by December 2005, 5.75% by December 2010, and 10% by 2020 of all petrol and diesel transport fuels should be biomass-derived. This directive has the aim to ensure the security of European energy supply, environmental sustainability, and the achievement of the Kyoto Protocol targets (Ragauskas *et al.* 2006).

In Italy, the "Biofuel Directive" has been enforced through the 128/2005 legislative decree. In 2007, Italy produced $60*10^6$ liters of bioethanol, production rate that was kept also in 2008 (EBIO 2009). In other countries the total amount has increased over time. Besides, the most part of Italian bioethanol production is intended for export. Thus, in spite of the European policy on renewable fuels, Italy lags behind with most investments focused on hydroelecticity, and other sustainable energy sources such as sun, wind and biomasses for combustion, other than biofuels.

The production of biofuels have been boosted through government subsidies, thus far. In 2005, for example, bioethanol was not cost competitive with petroleum-based fuels without subsidy, given the current prices and technology. In the same year, ethanol net production cost was $0.46 per energy equivalent liter (EEL) of gasoline, while wholesale gasoline prices averaged $0.44/liter. Further increases in petroleum prices above 2005 average prices would improve the cost competitiveness of biofuels. As a matter of fact, at the moment biofuels become competitive with regards to fossil fuels mainly benefiting from government subsidies, even though oil prices reach high values. For istance, in the U.S. the federal government provides $0.20 per EEL for ethanol. Besides, in the U.S., biofuels producers also benefit from federal

crop subsidies that lower prices of the raw materials (i.e.: corn prices are approximately half of ethanol production's operating costs).
Fossil energy use imposes environmental costs not captured in market prices, so whether a biofuel provides net benefits to society depends not only on its cost competitiveness but also on its environmental costs and benefits in comparison with its fossil fuel alternative. On the other hand, subsidies for economically uncompetitive biofuels are justified if their life-cycle environmental [i.e.: positive output/input energy ratio, lower Greenhouse gas (GHG) emissions (CO_2, CH_4, N_2O-)] impacts are sufficiently less than for alternatives.

1.2 First generation bioethanol

In the field of biofuels, much research has been devoted to the implementation of first generation bioethanol, produced from easily fermentable raw materials, such as corn, sugar cane, sugar beet. The inherent major concern for this biofuel is related to the use of materials otherwise exploited as food, with the consequent reduction in food supply and increase in its costs. As a matter of fact, in the U.S. the price of a bushel of corn has more than doubled between 2006 and 2008 and, as it has been also affirmed by the chief of the World Bank Robert Zoellick, the "demand for ethanol and other biofuels is a "significant contributor" to soaring food prices around the world". Besides, as far as environmental aspects are concerned, there is a huge debate on whether first generation fuels use provides environmental and energetic advantage in comparison to fossil fuels exploitation. Hill *et al*. (2006) asserted that the production and use of corn bioethanol release 12% less net GHG emissions than production and combustion of an energetically equivalent amount of gasoline. Differently, according to a study by Liska *et al.* (2009), corn bioethanol GHG emissions were estimated to be from 48 to 59% less as compared to gasoline. Besides the questionable reduction in GHG emissions, the convenience of producing ethanol from corn is also put into doubt by energetic balances. The Net Energy Balance (NEB) index, that stands for the difference between the energy needed for the production of a fuel and its energetic content, is only slightly positive for first generation bioethanol. Actually, it provides only 25% more energy than required for its production (Hill *et al*., 2006).
Statements about energetic and environmental impacts can improve if the life-cycle analysis provides for the further possible exploitations of coproducts, as performed in the so called closed-loop system (Liska *et al*., 2009). GHG emissions are reduced till 67%, considering that all coproducts distiller grains are consumed at a cattle feedlot next to the ethanol production plant.

1.3 Second generation bioethanol

In Sepember 2008, the European Parliament's industry and energy committee, besides confirming the 10% target by 2020, asked for at least 40% of this goal to be met by "non-food and feed-competing" second generation biofuels or green electricity and hydrogen (http://www.euractiv.com/en/energy/biofuels-generation/article-165951). The so-called second generation bioethanol is produced from ligno-cellulosic materials, thus avoiding the conflict between feedstocks uses for fuel and food production. Among the available resources exploitable for second generation bioethanol there are all those residues coming from different human activities such as agriculture (i.e.: cereals straws), food processing-industry, forestry, besides green

and organic fraction of urban wastes. All these lignocellulose containing materials are widely produced along the year in many countries.
As it has been stated by prominent authors, large-scale use of ethanol as fuel will almost certainly require cellulosic technology (Farrell *et al*., 2009). Actually, the exploitation of all these resources could guarantee the achievement of the fixed production targets, otherwise impossible to attain. As a matter of fact, available areas for corn, sugar cane or dedicated crops are limited and, as in the case of corn, it would be impossible to divert the whole harvest only to biofuels production.
As far as environmental and energetic balances are concerned, even the global conservation organisation Wold Wildlife Fund (WWF) acknowledges that the use of lignocellulosic materials for energy production will allow higher yields, greater $C0_2$ emission savings, requiring fewer inputs than agricultural energy crops such as sugar beet, oil seed rape or wheat (http://www.euractiv.com/en/energy/biofuels-generation/article-165951#section-1).
Besides, the large exploitation of cellulosic raw materials will require the implementation of efficient pretreatment techniques. As a matter of fact, the main drawback of using lignocellulose is related to the complexity of plant cells structure and to its chemical components.

1.4 Worldwide bioethanol production plants

As reported in table 1.1, different plants are now producing bioethanol from different raw materials worldwide. Most of them such as Agrana, use cereals; some others exploit cereal straws (Iogen Corporation, Canada and Abengoa, Spain). Broin jointly with U.S. DoE, DuPont and Novozymes in Iowa (U.S.) use corn fiber and stover. Whereas, Sekab exploits forestry products in Sweden.
In Italy, the first plant for the production of second generation bioethanol is being built by the italian Mossi & Ghisolfi group and it will use agro-energetic crops.

Company	Location	Raw materials
Iogen Corporation	Canada	Wheat, oat and barley straw
Abengoa Bioenergy	Europe (Spain) USA	Grain, Dried Distilled Grain Wheat/barley straw
Broin jointly with U.S. DoE, DuPont and Novozymes	USA Iowa	Corn fiber and stover
Verbio Vereinigte BioEnergie AG	Germany	Grain Rapeseed oil
Agrana	Austria Hungaria	Cereals
Tereos	France, Czech Republic, Brazil	Sugar beet, wheat and sugar cane
Inbicon	Denmark	Wheat straw
Sekab	Sweden	Forestry products

Table 1.1: World wide bioethanol production plants.

1.5 Lignocellulose composition

The main lignocellulose costituents are lignin, cellulose, hemicellulose and pectin. Plant cells (fig. 1.2) have a rigid wall surrounding the plasma membrane, called cell wall.

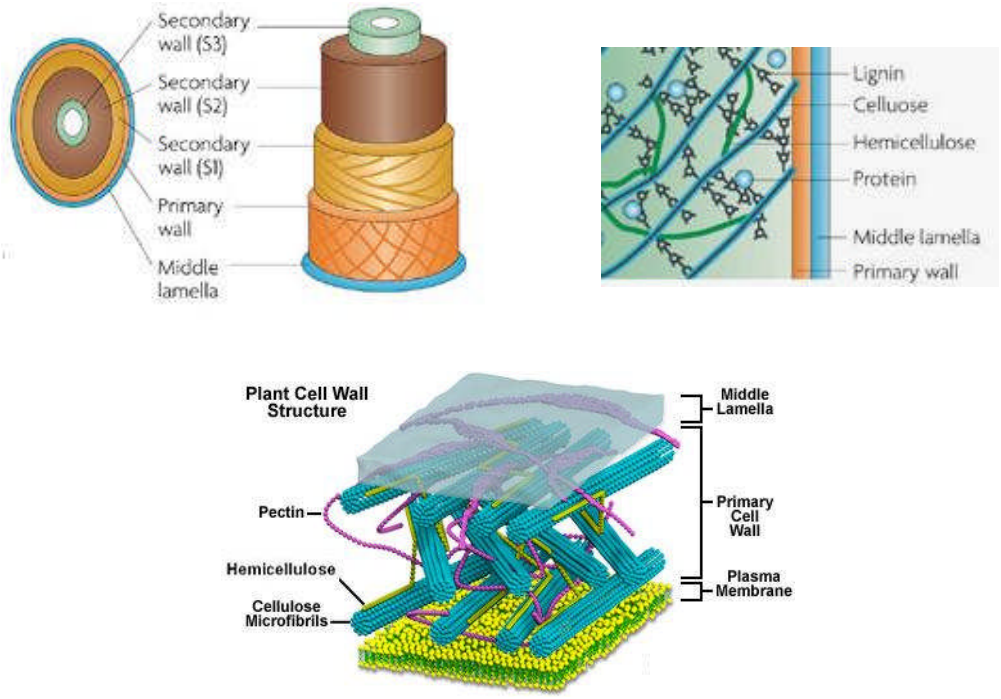

Figure 1.2: Schematic view of plant cell wall structure. The different levels of organization, the components and their position in the plant cell wall structure.

It is a very complex structure and serves a variety of functions, from protecting the cell to regulating the life cycle of the plant organism. Many plant cells have both a primary cell wall, and a secondary cell wall they develop inside the primary wall after the cell has stopped growing. The main components are cellulose, hemicellulose, pectin and lignin.

Botanics classify plants into two main families, namely monocotyledons and dicotyledons, differing for macromolecular composition and for hemicellulose and pectin chemical structures. Dicotyledons hard-wood have the following composition: cellulose: 40-50%, lignin: 15-25%, hemicellulose: 15-25%. Peach, apple and tomato, also belong to the family of dicotyledons, showing the following average composition: 30% of cellulose, 30% of hemicellulose, 35% of pectin and 1-5% of structural proteins. For this family a model of stucture has been proposed by Vincken *et al.* (2003) as reported in figure 1.3, elaborated by Vorwerk *et al*., (2004). The essential features of the model are that the cellulose microfibrils are crosslinked by

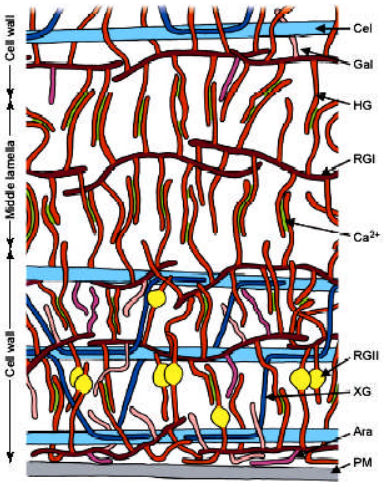

Figure 1.3: Model of the organization of polysaccharides in plant cell walls. (PM= plasma membrane; Ara= Arabinans; XG= xyloglucan; RGII= ramnogalacturonan II; RGI= ramnogalacturonan I; HG= homogalacturonan; Gal=galactans; Cel=cellulose).

hemicellulose, primarily xyloglucan, which is hydrogen bonded to the cellulose fibrils. Rhamnogalacturonan I is also aligned with the cellulose microfibrils. Homogalacturonan, arabinans, galactans and rhamnogalacturonan II are envisioned as being side branches attached to a high molecular weight RGI backbone.

Cellulose

Cellulose is the most abundant polymer on Earth, accounting for 50% of the biomass nature produces through photosynthesis. Cellulose is a long linear polymer with a degree of polymerization of at least 15,000 D-glucose units. Glucose monomers are linked together with β-1,4-glycosidic bonds to form highly stable chains, and these latter further aggregate together via hydrogen bonds to form a rigid crystalline structure that is water-impermeable, water insoluble and little accessible to enzymatic hydrolysis (Linko, 1987). Cellulose fibrils can even be found in regions of less ordered and thus more accessible amorphous structure. Although chemically simple, the intermolecular bonding pattern can result in a very complex morphology (Hon, 1994).

Hemicellulose

Xylan is the main component of hemicelluloses, and it is built by β-1,4-linked xylopyranosyl residues. In contrast to cellulose the xylan structure is very variable, depending upon its source. The structure ranges from almost linear unsubstituted chain, e.g. in some grasses, to highly branched heteropolysaccharides in cereal seeds. The prefix “hetero“ indicates the presence of sugars other than D-xylose, mannose, arabinose. The main chain is usually substituted to various degree by residues of 4-O-methyl-D-glucuronic acid, D-glucuronic acid, L-arabinofuranose and in some cases is also esterified by acetyl groups. The substituents may be also constituted by oligosaccharides and can be esterified by cinnamic (phenolic) acids, such as ferulic acid. To hemicelluloses family also belong other polymers such as xylomannan, glucuronoxylans, mannans, glucans and galactans.

Pectin

The word pectin includes a family of complex polysaccharides with high molecular weight (20-400 kDa), with acidic features. From a chemical point of view, it is constituted by monomeric units of D-galacturonic acid joined together by α-1,4 linkages; this chain also comprises units of 1,2-L-rhamnose. In pectin structure D-galactose, L-arabinose, D-xylose and L-fucose are also present, forming branches in the molecule. Whereas the coextensive network of pectins provides the cell wall with the ability to withstand compression, the cross-linking hemicellulose increases the tensile strength of the cellulose and it is organized into a network with the cellulose microfibrils.

Lignin

The secondary plant cell wall, often deposited inside the primary cell wall as the cell matures, commonly contains lignin, that imparts considerable strength to the structure of the secondary wall. Lignin is formed by oxidative coupling of primarily 4-hydroxycinnamyl alcohols (Ralph *et al*., 2004), such as trans-*p*-coumaryl, transconiferyl, and trans-sinapyl alcohols (Sarkanen *et al*., 1971) (Fig. 1.4).

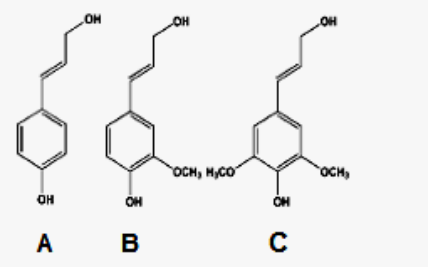

Figure 1.4: Lignin primary precursors: trans-*p*-coumaryl (A), transconiferyl (B) and trans-sinapyl (C) alcohols

These lignols are incorporated into lignin in the form of the *phenylpropanoids p*-hydroxyphenyl (H), guaiacyl (G), and syringal (S), respectively. *Gymnosperms* have a lignin that consists almost entirely of G with small quantities of H. Different plant species have different proportions of the three groups. For example, *dicotyledonous angiosperms* often present only G and S (with very little H), and *monocotyledonous* lignin a mixture of all three. Whereas woody plants and dicotyledons have rigid lignified cell walls, monocotyledons, like grasses, have lignified cell walls as well as walls rich in low molecular weight phenolic acids (*p*-coumaric and ferulic acids), ester-linked to arabinose. Due to its biosynthesis via random free-radical condensation, no unique structure can be established, and as a matter of fact, lignins can have multiple structures. Lignin and other aromatics link with, and physically mask, the polysaccharides present in lignocellulose, protecting them from hydrolytic activities. Thus, their removal is necessary to free polysaccharides. However, due to its chemical composition and complex structure, lignin is characterised by a high recalcitrance, thus requiring very harsh conditions such as chemical or chemico-physical treatments.

1.6 Conventional process for bioethanol production

The typical process for bioethanol production from lignocellulosic materials conventionally includes a pretreatment unit, a saccharification step, an ethanol production phase and the unit of alcohol recovery by distillation.

1.6.1 Pretreatment practices

As before mentioned, one of the main drawbacks related to lignocellulose exploitation is its complex structure, with lignin and other heterogeneous polymeric molecules constituting a resistant barrier all around the polysaccharidic fraction. A pretreatment of the lignocellulosic material is necessary to remove/reduce the barrier made by both lignin and hemicelluloses. Furthermore, the physical and/or chemical pretreatment may increase the accessibile surface area and change the crystallinity of the cellulose, which results in increased digestibility of the cellulose. It is, thus, required to set up appropriate pre-treatment processes. The ideal pretreatment should limit, as far as possible, the need for reducing the size of biomass particles, preserve the pentose (hemicellulose) fractions, limit the formation of degradation compounds that would inhibit the growth of fermentative microorganisms, reduce energy demands and costs. These features, along with low pretreatment catalyst cost or inexpensive catalyst recycling and generation of higher value lignin co-products, form the basis for comparison of various pretreatment options (del Campo *et al*., 2006). A common way adopted for loosening lignocellulose structure is the acid/alkali hydrolysis with concentrated or diluted sulphuric acid or soda (Lee, 2005, Silverstein *et al*., 2004). Diluted acid pretreatments are used to convert lignocellulosic biomass, including hemicellulose fraction, into soluble sugars releasing unaffected the cellulose fraction (Lee *et al*., 1999). On the other hand, the alkali treatment can swell the lignocellulose structure, disrupt lignin and reduce cellulose crystallinity (Li *et al*., 2004). Besides, it removes the acetyl and uronic acid substitutions on

hemicellulose lowering the accessibility of the enzyme to cellulose and hemicelulose surfaces (Ramirez, 2005). Both processes entail the use of chemical solvents, the formation of inhibitory compounds and the release of polluting waste waters to be treated before their disposal or reuse. Other strategies incude physical methods or a combination of chemical and physical ones. Steam explosion, also called autohydrolysis, makes use of high temperatures and pressures to disrupt biomass internal structures (Mosier *et al.*, 2005). The biomass can be held at high temperature/pressure in order to promote hemicellulose hydrolysis, releasing free sugars. This process is thought to be due to the acids that are produced during the process, or to water that in appropriate conditions can act as an acid (Mosier *et al.*, 2005). Steam explosion must be stopped otherwise glucose and xylose degradation products can be obtained.

1.6.2 The saccharification step

Cellulose hydrolysis can be catalysed either by diluted/concentrated acid or enzymes. Sulfuric acid use has, as main drawback, the fact that it also readly degrades glucose at the high temperatures required for cellulose hydrolysis.
Enzymes have many advantages such as very mild operative conditions, high yields and no corrosion problems.
However, in order to exploit enzymatic hydrolysis at industrial scale, a huge amount of hydrolytic enzymes is required, and enzymes production costs are still too high for economical production of fuel ethanol from biomass.
Different classes of enzymes have to take part in the reaction to accomplish the complete saccharification of the cellulose present in the raw material. They belong to four different classes: the endo-cleaving (endoglucanases), exo-cleaving (exocellulases, cellobiohydrolases), that act directly on the polymer, the β-glucosidases and cellobiose dehydrogenase that act on the resulting cellobiose or cello-oligosaccharide hydrolysing it or subjecting it to dehydrogenation, respectively.

Endo-1,4-β-glucanase (EC 3.2.1.4, endocellulase)

Endo-1,4-β-glucanases catalyse the cleavage of internal glycosic bonds, showing a preference for amorphous regions of cellulose. However, significant activity towards crystalline cellulose was found only for the *Irpex lacteus* cellulase, the Cel5A *Gloeophyllum trabeum* and EG35 from *Fomitopsis palustris* (Kanda *et al*., 1976; Cohen *et al*., 2005; Yoon *et al*., 2007). The latter two enzymes belong to a group of processive endoglucanases, originally reported from cellulolytic bacteria. These enzymes cleave cellulose internally but also release soluble oligosaccharides before detaching from the polysaccharide and thus act as a combination of endoglucanase and cellobiohydrolase (Tomme *et al*., 1996; Gilad *et al*., 2003). The enzymes are monomeric, with molecular masses typically between 22 and 45 kDa but enzymes with almost double size were found in *Sclerotium rolfsii* and *Gloeophyllum sepiarium* (Sadana *et al.*, 1984; Bhattacharjee *et al*., 1993).

Cellobiohydrolase (CBH, EC 3.2.1.91; exocellulase)

Cellobiohydrolases are widespread among fungi. They are tipically monomeric non glycosilated proteins with molecular masses ranging from 55 and 65 kDa. They are typically divided into two categories, those that cleave cellulose by its reducing ends, and those that act from the non-reducing ones. This particular behaviour is maybe the answer for the high number of cellobiohydrolases found in many different fungi (Uzcategui *et al*., 1991). Cellobiohydrolases are typically active on crystalline

cellulose, e.g. Avicel. Interestingly, CBH58 and CBH50 from *Phanerochaete chrysosporium* are not active on carboxymethylcellulose and CBH62 as well as both cellobiohydrolases from *Pleurotus ostreatus* exhibit only weak activity against this substrate (Uzcategui *et al*., 1991b; Garzillo *et al*., 1994). Cellobiohydrolases are also active on cellotriose, cellotetraose or higher cellodextrins (Kanda *et al*., 1989; Schmidhalter *et al*., 1993; Hishida *et al*., 1997). Not surprisingly, cellobiose acts as a competitive inhibitor of this class of enzymes.

β-Glucosidase (EC 3.2.1.21)
Because cellobiose is a largely available substrate, β-glucosidases are produced by the majority of microorganisms (Lynd *et al*., 2002). As before mentioned they act on cellobiose or cello-oligosaccharides freeing glucose units. β-glucosidases isolated so far exhibit high structural variability, partly reflecting the intracellular/extracellular localization of the enzyme. Thus, β-glucosidases can be extracellular, cell wall-associated and intracellular. The mycelia-associated fraction in *Pleurotus ostreatus*, *Trametes versicolor* and *Piptoporus betulinus* accounted for 65%, 13%, and 35% of the total activity, respectively (Valaskova *et al*., 2006).

Cellobiose dehydrogenase (CDH; EC 1.1.99.18)
CDH is an extracellular enzyme produced by basidiomycetes and ascomycetes. It efficiently oxidizes cellobiose but also soluble cellodextrins, mannodextrins and lactose to their corresponding lactones using a wide spectrum of electron acceptors including quinones, phenoxyradicals, Fe^{3+}, Cu^{2+}, cytochrome c (Henriksson *et al*., 2000; Zamocky *et al*., 2006).

1.6.3 Fermentation of biomass hydrolyzates
The classic configuration employed for fermenting biomass hydrolizates involves a sequential process where the steps of hydrolysis of polysaccharides and alcohol production, through fermentation, are carried out in two separate vessels (Separate Hydrolysis and Fermentation, SHF) (Sanchez *et al*., 2008). In the alternative variant, the hydrolysis and fermentation are performed in a single unit (Simultaneous Saccharification and Fermentation). The most employed microorganism for fermenting lignocellulosic hydrolyzates is *Saccharomyces cerevisiae*, which ferments the hexoses contained in the substrate but not the pentoses. In alternative, the ability of several microorganisms of using pentoses, such as *Pichia stipitis* and *Zymomonas mobilis* can be exploited, to increase ethanol yield. In the SHF process, solid fraction of pretreated lignocellulosic material undergoes hydrolysis. Once this phase is completed, the resulting cellulose hydrolyzate is fermented and converted into ethanol. One of the main features of the SHF is that each step can be performed at its optimal operating conditions. However, if cellulolytic enzymes are inhibited by end products of the hydrolysis, the simoultaneous process can be applied, in which the concomitant production of sugars-saccharification- and their microbial conversion into ethanol -fermentation- is accomplished in a single tank. It has many advantages over the SHF such as higher ethanol yields and less energetic consumption. However, this process operates at non-optimal conditions for hydrolysis and requires a higher enzyme load, that positively influences substrate conversion but negatively process costs.

1.7 Bioethanol production: an alternative approach

1.7.1 Alternative pretreatments

A more environmental friendly solution, that has found increasing application in the pretreatment step, is the use of biological systems, such as microorganisms or their lignocellulolytic activities. Several enzymes have to be present (ligninases, xylanases, pectinases), to selectively remove the different macromolecular components, thus reducing the complexity of these matrices, and allowing enzymes to reach the polysaccharidic core.

Lignin modifying enzymes -LME-

Lignin modifying enzymes (LME) are all those activities cooperating to depolymerize lignin, even if with different relevance, such as laccases, manganese peroxidases (MnP), lignin peroxidases (LiP), veratryl alcohol oxidases (VAO).

Laccases

Laccases, EC 1.10.3.2, *p*-diphenol:dioxygen oxidoreductase, are multicopper enzymes, catalysing the one-electron oxidation of four substrate equivalents, with the concomitant reduction of O_2 to water (Fig 1.5a). They are ubiquitous in nature and they have been found in fungi, plants and insects. The physiological function of these biocatalysts, which can be secreted or intracellular, is different in the various organisms but they all catalyse polymerization or depolymerization processes (Riva, 2006). It has been proposed that laccases take part in the formation of fruit bodies, the production of conidial pigments, pathogenesis, and both in the polymerization and depolymerization of lignin. The catalysis carried out by all members of this family, is guaranteed by the presence of different copper centres, classified on the basis of their spectroscopic properties: one type-1 (T1) copper, one type-2 (T2) and two type-3 (T3) copper ions (Fig. 1.5b).

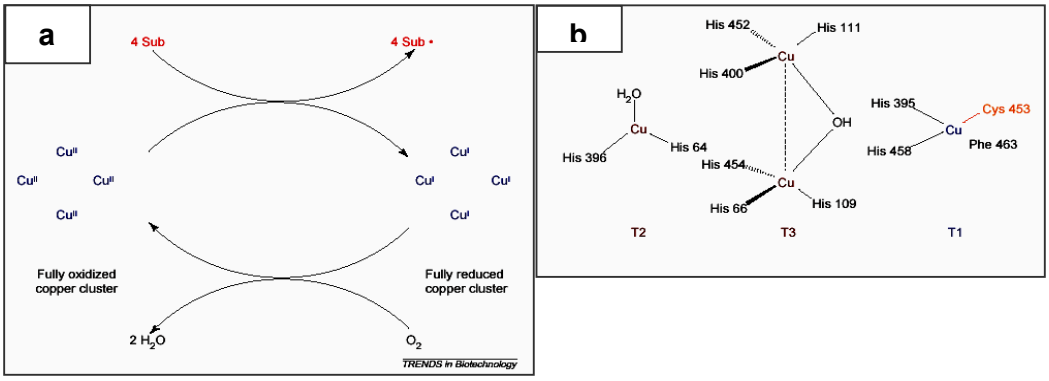

Figure 1.5: Laccases: active-site structure and catalytic cycle. (a) Schematic representation of a laccase catalytic cycle (Claus, 2004). (b) Model of the catalytic cluster of the laccase from *Trametes versicolor* made of four copper atoms. (Piontek *et al.,* 2002). Sub: substrate molecule; Sub*: oxidized substrate radicals.

Laccases are interesting enzymes since they are able to oxidase a wide range of phenolic and, in the presence of appropriate redox mediators, non-phenolic compounds because of the high redox potential of T1 copper: the site where substrate oxidation takes place. Laccases are usually secreted in multiple isoforms, depending on culture condition and state of development (sporocarp, mycelium, etc.).

The various isoforms usually differ for molecular mass, pI, glycosylation pattern and can be induced by different culture conditions.

Manganese peroxidase (MnP)
Manganese peroxidase (MnP) is a heme glycoprotein that catalyses the oxidation on Mn^{2+} to Mn^{3+} in the presence of H_2O_2. Mn^{3+} is efficiently stabilised in aqueous solution by α-hydroxy acids (Wariishi *et al.*, 1992). Chelated Mn^{3+} acts as a highly reactive (up to 1510mV) low molecular weight diffusible redox-mediator. Thus, MnP are able to oxidase and depolymerise their natural substrate, i.e., lignin as well as recalcitrant xenobiotics, such as textile dyes (Moldes *et al*., 2003).

Lignin peroxidases
Lignin peroxidases (LiP, EC1.11.1.13) are N-glycosylated enzymes which catalyzes the oxidation of non-phenolic aromatic lignin moieties and similar compounds by one-electron mechanism to form reactive radicals. They contain heme in the active site and show a classical peroxidase mechanism. The role of LiP in ligninolysis could be the further transformation of lignin fragments which are initially released by MnP.
LiP are not essential for the attack on lignin: several highly active White Rot Fungi and litter-decaying fungi do not produce this enzyme.

Veratryl alcohol oxidase (VAO)
Veratryl alcohol oxidases catalyse the reduction of O_2 to produce H_2O_2, with the formation of veratrylaldehyde. It can take part in the lignin degradation process either reducing the radical intermediates formed by laccase, MnP and LiP, thus preventing their repolymerization, or producing hydrogen peroxide for MnP and LiP action (Marzullo *et al.*, 1995).

Glycosyl hydrolases
The glycosyl hydrolases putatively involved in lignocellulose pretreatment include hemicellulose and pectin hydrolysing enzymes. They take part in the removal of the barrier of pectin, and in the hydrolysis of hemicellulose that can be linked to the lignin fraction to a variable extent.

Xylanases
Xylanolytic enzymes have been found in bacteria, fungi, yeasts and protozoa (Dekker *et al*., 1976, Eriksson *et al*., 1990). Most, if not all, fungal plant pathogens produce and secrete enzymes that degrade cell wall hemicellulose (Prade, 1995). Xylanolytic enzymes occur also in plants, where these enzymes participate in the process of cell wall extension, cell division, seed germination and other morphological and physiological events in plants. Depending on its complexity, the complete breakdown of xylans requires the action of several enzyme components which can be considered as a xylanolytic system. The crucial enzyme for xylan depolymerization is endo-β-1,4-xylanase (β-1,4-xylan xylanohydrolase; EC 3.2.1.8, abbreviated EX). The main chain substituents are liberated by the corresponding glycosidases or esterases: α-L-arabinosyl residues by α-L-arabinofuranosidases (α-L-arabinofuranoside arabinofuranosidase; EC 3.2.1.55), D-glucuronosyl and 4-O-methylglucuronosyl residues by α-glucuronidase (EC 3.2.1.139), acetic acid, ferulic acid and p-coumaric acid residues by the corresponding esterases (EC numbers not assigned).

Endo-β-1,4-xylanase (EC 3.2.1.8) (Ex)

Endo β-1,4-xylanases attack xylan backbone, and produce xylo-olygosaccharides with free reducing ends, catalyzing the following reaction:

$$R_1\text{-Xyl}\beta1\text{-4Xyl}\beta1\text{-4Xyl}\beta1\text{-4Xyl-}R_2^* \xrightarrow{H_2O} R_1\text{-Xyl-}\beta\text{-1,4Xyl}^* + \text{Xyl-}\beta\text{-1,4-Xyl-}R_2^*$$

R_1 and R_2, substituted or unsubstituted portions of the main chain of xylan;
*: Reducing end

β-Xylosidase (xylobiase or exo-β-1,4-xylanase) (EC 3.2.1.37)

β-Xylosidase hydrolyzes xylan, xylobiose and higher linear β-1,4-xylooligoaccharides to monomer, acting at the carbohydrate sides and also releases xylose from branched or substituted xylooligosaccharides produced by the action of Exs. The chemical reaction catalyzed is as follows:

$$\text{Xyl-}\beta\text{-1-4Xyl} \rightarrow 2\ \text{Xyl} \quad \text{or} \quad \text{Xyln}_n \rightarrow \text{Xyl}_{n-1} + \text{Xyl}$$

Acetylxylan esterase (3.1.1.72)

Acetylxylan esterases (AcXEs) are able to remove acetyl groups esterifying D-xylopyranosyl residues of xylan main chain at positions 2 or 3 (as reported in the following scheme). AcXEs deacetylate also partially acetylated xylooligosaccharides.

$$R_1\text{-Xyl}\beta1\text{-4Xyl}\beta1\text{-4Xyl}\beta1\text{-4Xyl}\beta1\text{-4Xyl-}R_2^* \xrightarrow{H_2O} R_1\text{-Xyl}\beta1\text{-4Xyl}\beta1\text{-4Xyl}\beta1\text{-4Xyl}\beta1\text{-4Xyl-}R_2^* + 2\ \text{AcH}$$

(2-Ac, 3-Ac)

*: Reducing end

The enzyme action on polysaccharide substrates creates new sites on the xylan main chain, suitable for productive binding with depolymerizing EXs. Acetylxylan degradation with EXs proceeds faster and to a higher degree in the presence of AcXEs. Deacetylation of xylooligosaccharides makes the oligosaccharides fully susceptible to the action of β-xylosidase.

α-L-Arabinofuranosidase (EC 3.2.1.55)

α-L-Arabinofuranosidases participate in the hydrolysis of plant polysaccharides build from or containing non-reducing terminal α-L-arabinofuranosyl residues (Kormelink, 1992). They are responsible for the removal of arabinose molecules present along the backbone.

$$\text{Xyl}\beta1\text{-4Xyl}\beta1\text{-4Xyl-R} \quad (\text{3 (2), α1, Ara}f) \rightarrow \text{Xyl}\beta1\text{-4Xyl}\beta1\text{-4Xyl-} + \text{Ara}$$

$$\text{R'-Xyl}\beta1\text{-4Xyl}\beta1\text{-4Xyl-R} \quad (\text{3(2), α1, Ara}f) \rightarrow \text{R'-Xyl}\beta1\text{-4Xyl}\beta1\text{-4Xyl-R} + \text{Ara}$$

Other natural substrates of α-L-arabinofuranosidase are glycosylated terpenols of grape, important precursors of grape and wine aromas.

Pectinolytic enzymes

Pectinolytic enzymes catalyzing the degradation of pectic substances are of great industrial importance (Spanga *et al.*, 1995). Pectinolytic enzymes are classified according to their mode of attack on the galacturonan part of the pectin molecule. They can be distinguished from pectin methylesterases (EC 3.1.11.1), that de-esterify pectins to low methoxyl pectins or pectic acid, and from pectin

depolymerases, that split the glycosidic linkages between galacturonosyl (methyl ester) residues. Polygalacturonases split glycosidic linkages next to free carboxyl groups by hydrolysis while pectate lyases split glycosidic linkages next to free carboxyl groups by β-elimination. Both endo types of Pgs and PAls (EC 3.2.1.15 and EC 4.2.2.2, respectively) are known to randomly split the pectin chain. Exo-Pgs (EC 3.2.1.67) release monomers or dimers from the non-reducing end of the chain, whereas exo-PAls (EC 4.2.2.9) release unsaturated dimers from the reducing end. Highly methylated pectins are degraded by endo-pectin lyases (Pl; EC 4.2.2.10) and also by a combination of pectin esterases with Pg or PAl (Silva *et al*., 2002).

1.8 Main issues and future challenges for the second generation bioethanol production

The elements that constitute crucial economic and process bottlenecks of second generation biofuel production process are feedstocks and conversion enzymes. In order to reduce total bioethanol production costs, an integrated approach should be applied, in which economically and environmentally sound strategies are followed. In the following sections the two main issues affecting total bioethanol price are addressed, and a new approach, that of biorefinery, for the improvement of cost competitiveness will be briefly described.

1.8.1 Waste lignocellulosic materials

Since almost half of the total cost of second generation fuels is due to raw materials (Lynd, 2008), exploiting the potential of lignocellulosic wastes would contribute to lowering total process costs. Worldwide, lignocellulosic wastes (mainly, agricultural, food farming residues and organic and green fractions of urban wastes) are produced in high amounts and their disposal is a major problem for their producers and public authorities. Usually, these materials are treated as wastes and disposed of as such, either by burning, landfilling or by using them as feed for animals. These management practices cause environmental and economical problems given the high costs for their transport, the low calorific power and high water content, and the evidence that not every material is appropriate for feeding every animal. Besides, due to legislation and environmental reasons, the industry is increasingly being forced to find an alternative use for its residual matter, thus triggering the reutilization of biological wastes in economically and ecologically sustainable ways. Furthermore, government policies are boosting the development of processes for renewable energies production that are based on locally available resources, avoiding the dependence on elsewhere produced feedstocks (i.e.: petrol), thus reducing total economical and environmental costs due to oil exportation and to the immission of CO_2, respectively. In the northern countries of Europe the main available resources are forestry residues, and as a matter of fact, much research has been focused on their exploitation in processes for bioethanol production (Galbe *et al*., 2002). Differently, in those areas, such as the Mediterranean basin, where agriculture and food industries are widespread, residues coming from these activites have a high practical potential, unlike forestry ones. Furthermore, in the same areas people have the habit of using fresh foods instead of already cooked and packed stuff, so that potentially available amounts of organic fraction of urban wastes (vegetables, fruits, wood and urban green pruning residues) are bigger than in the northern and western countries. Thus, organic fraction of municipal wastes could become a resource,

rather than a problem. However, it is well known that the separate collection of rubbish is more widespread in the northern countries, than in the southern ones.

1.8.2 Alternative ways for lignino-cellulolytic enzymes production

Enzymes are the second element contributing to soaring second generation fuels costs, roughly doubling the cost of cellulosic ethanol production and lessening the economic advantages of using waste materials (Sanchez *et al.*, 2008). Hence even if more environmental friendly, enzymatic treatment results to be economically less convenient than conventional chemical methodes (alkali-, or acid treatments). Thus, strategies to improve whole process economicity could provide for the reduction of enzymes costs either through their production with fermentative techniques which are more economically advantageous (characterized by low costs, high product yield), or improvement of enzymatic catalytic parameters (based on genetic manipulation).
In this section only the first proposed approach will be analysed. A valide alternative to the use of purified/commercial enzymes may be the direct production of the enzymes of interest on the lignocellulosic material to be converted, through its microbial fermentation. A powerful technique for exploiting lignocellulosic waste materials is solid state fermentation (SSF). It reproduces natural microbiological processes, such as composting and ensiling. SSF stands for those processes in which microorganisms grow in the absence or near-absence of free water (Pandey, 2003; Pandey *et al*., 2000), using lignocellulosic materials not only as a support, but also as a source of carbon and energy. Given the environmental conditions, this process appears really close to the natural environment in which many higher filamentous fungi have evolved. These microorganisms have long been exploited since ancient times, and still they are, for their ability to produce interesting compounds such as antibiotics, colorants, flavours and valuable, industrially relevant enzymes (i.e.: ligninases, cellulases, hemicellulases, proteases, etc.). From another point of view, lignocellulosic wastes may contain significant concentrations of soluble carbohydrates and inducers of enzyme synthesis ensuring efficient production of ligninocellulolytic enzymes (Jaszek *et al.*, 1998; Mamma *et al.*, 2008; Sabu *et al*., 2005). Solid state fermentation offers several advantages over submerged fermentation, among which high volumetric productivity, low capital investment and energy requirement, ease of product recovery, less waste-water output, lower catabolite repression. Its main drawbacks, on the other hand, are the build-up of gradients in temperature, pH, moisture, substrate concentration or pO_2, which are difficult to control under limited water availability. To overcome all of them, a considerable amount of work has been done in recent years to understand the biochemical and engineering aspects of SSF processing (Pandey, 2003, Mitchell *et al*., 2006). Since the last decade, many studies about the application of SSF were focused on adding an extra value to agro-industrial residues and several processes have been developed aimed at enzyme production and metabolite synthesis (Rodiguez-Couto, 2008; Laufenberg *et al.*, 2003, Pandey *et al.*, 1999). Enhancing the value of lignocellulosic feedstocks and exploiting their potential is the goal of the so-called ‘upgrading concept’ (Laufenberg *et al.*, 2003). Possible products are enzymes, microbial biomass (i.e.: edible mushrooms), sugars for fuel ethanol production, aromatic compounds (vanillin, fruity flavours), polyphenols, food/feed additives (fibres, vitamins), organic acid (lactic and citric acid), biocolorants, bulk chemicals (ethylene, propylene glycol, isopropanol, acetone, butylene).

1.8.3 The biorefinery approach

The overall profitability and productivity of bioethanol can be improved by integrating the production of other marketable bioproducts into the fuel production process. This strategy puts into practice the goal and strategy of the biorefinery concept. A modern biorefinery parallels the petroleum refinery (Ragauskas, 2006) in that an abundant raw material, consisting primarely of polysaccharides and lignin, enters the biorefinery and, through an array of processes, is fractionated and converted into a mixture of products including, besides transportation fuels, co-products and direct energy. In a typical petroleum refinery plant almost 5% of the total petrol output goes to chemicals, and the remaining part to the production of fuels and energy. As pointed out by Kamm (2004) this ratio is not supposed to change in modern biorefinery. However, the commercialization of biomass-based biorefineries as viable substitutes for (petro)chemical processes is highly dependent on the exploitation of the full potential of biomass in order to recover and/or trasform a spectrum of complex organic macromolecules (e.g. carbohydrates, protein, oil, lignin) as well as many other chemical constituents (e.g. antioxidants, functional proteins, lipids). The exploitation of each one of these components would lead to the production of several products ranging from commodities (high volume – low market value) such as above reported biofuels and biodegradable plastics, to platform molecules (intermediate volume and market value) such as succinic acid and lactic acid, and speciality chemicals (low volume – high market value) such as functional ingredients, colorants, flavours and pharmaceuticals. Till now several processes have been developed that use bio-based feedstocks for the production of fragrances, solvents, plastics (such as polylactic acid as bioderived plastic) (Auras *et al*., 2004). In order to better contribute to the improvement of bioethanol production process, economic analyses and evaluations have to be done, in order to highlight the products with the highest market potential. The sugars rather than being transformed into ethanol, can be processed into building block chemicals (molecules with multiple functional groups that possess the potential to be transfomed into new families of useful molecules) by fermentation as well as by enzymatic and chemical processes. The key ones are C3 to C6 carboxylic acids, and alcohols such as, glycerol and sorbitol, as pointed out by the U.S. Department of Energy in a comparative study tab. 1.1).

Building blocks	
1,4 succinic, fumaric and malic acids	Itaconic acid
2,5 furan dicarboxylic acid	Levulinic acid
3 hydroxy propionic acid	3-hydroxybutyrolactone
Aspartic acid	Glycerol
Glucaric acid	Sorbitol
Glutamic acid	Xylitol/arabinitol

Table 1.1: Sugar-based molecules envisaged as promising chemical building blocks

Furthermore, to make the biorefinery approach widely applicable, the lignin content of lignocellulosics must also be addressed. As a matter of fact, applying different approaches lignin can be transformed into fuels (condensable gases, non condensable ones to be steam-reformed to synthesis gas -syngas-), chemicals (low molecular weight phenolic compounds) (Shabtai *et al.*, 2003). In addition, maximizing

the usage of biomass components would lead not only to significant improvement of process economics but also to waste minimization.

1.9 Aim of the thesis

The objective of this research thesis was to set up microbial and/or enzymatic systems for the valorisation of ligno-cellulosic wastes thanks to the production of industrially relevant products (enzymes, fermentable sugars for fuel bioethanol production, chemical building blocks, etc.). The reserach activities have had four different targets:

i. Selection of lignocellulosic raw materials
   The first step has been the selection of lignocellulosic feedstocks that are produced in high amounts on local scale yearly. Available data have been collected on residues coming from several activites such as agricolture, food farming processes, and on organic fraction of urban solid wastes. Data concerning yearly produced amounts, chemical composition, and the alternative methods already existing for their reuse have been sought.
ii. Selection of process microorganisms
   At the same time, literature data on fungi able to degrade the different macromolecular components present in wastes have been collected. Fungi have been grouped according to their conversion abilities, inferred from changes in substrates macromolecular composition, and from levels of the correlated hydrolytic/oxidative enzymatic activities.
iii. Set up of a Solid State Fermentation process
   Given the characteristics of both selected matrices (solid nature, good water content, nutriens and sugars availability) and conversion microorganisms (mostly evolved to colonise wood or woody substrates), Solid State Fermentation (SSF) has been chosen as fermentative technique. The influence of several parametes (pH, humidity, particle dimension, substrate to support ratio, inoculum conditions) have been studied in order to meet the conditions allowing good fungal waste colonization and conversion.
iv. Definition of process conditions allowing the obtainment of high added value products
   SSF experiments have been performed with the two fungal strains, selected in this research project as the best lignin-cellulolytic ones, on the two lignocellulosic wastes that have been chosen. Fungal growth has been monitored determining the time course of different parameters such as weight loss and humidity changes, levels of oxidative and glycosyl hydrolysing activites, and of reducing sugars. Further studies have been performed aimed at the identification of valuable industrially relevant compounds, originally present in the wastes or produced by fungi, benefitting from mass spectrometry analyses.

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2. Results

2.1 Selection of lignocellulosic waste materials

2.1.1 Introduction

The first goal of this research project has been the selection of wastes to be used as raw materials in fungal solid state fermentation experiments.
Lignocellulosic waste materials of interest belong to the following three classes: i) agricultural residues, ii) food farming residues and iii) green and organic fractions of urban wastes.
Waste raw materials were chosen taking into account, as selection criteria produced volumes (section 2.1.1), periodicity of their production (section 2.1.2), existence of alternative ways for their disposal or reuse (section 2.1.3), macromolecular composition (section 2.1.4).
Aim was to select the most abundantly produced ligno-cellulosic residues, coming from those cultures and food farming factories that are widespread in Italy and on the territory of Campania. The choice of having the source of raw materials near the site of its transformation is crucial, since it allows reducing the environmental and economic costs of their transport, which also cause high CO_2 emissions.
Time of wastes production was also investigated, being the continuous providing of materials a crucial issue for a biorefinery plant to be economically sustainable.
As third selection criterium, the existence of alternative ways for their disposal or for their exploitation was applied.
Aim of the so-called upgrading concept is to give value to a material previously considered as a waste and, thus, disposed of accordingly. If lignocellulosic materials are already included in a production process, the proposed biotechnological alternative, in which they are further exploited and transformed, should create higher added value in comparison with the previous one.
Another important aspect that was taken into account is the composition of waste materials, namely in terms of relative abundance of different polymeric components, such as lignin, pectins, cellulose and hemicellulose. As a matter of fact, different proportions of each polysaccharide (hemicellulose, cellulose) can influence the ratio between hexose and pentose sugars obtainable in the sugar mixture to be further processed in a biorefinery plant. Moreover, it is crucial to consider the amounts of lignin and pectines, since they constitute a physical barrier to the access of fungal hydrolythic activities to polysaccharides (fig. 1.2)

2.1.2 Results

2.1.2.1 Wastes volumes

The seventh article of 22/97 Legislative Decree of Italian Law, with its following modifications and integrations, classifies wastes according to their origin in urban and special wastes, the latter ones being further classified into dangerous and not dangerous.
Agrarian residues lay among the non dangerous special wastes, together with byproducts coming from the food processing industries, such as those of fruit and vegetables. Straw, seeds, stalks and leaves are the main residues obtained during the recovery of the staple product of herbaceous and woody[1] cultivations whereas

[1] Herbaceous cultures: wheat, durum wheat, oat, maize (corn), rice, sunflower, soy, potato, tomato and artichoke; woody cultures: grapevine, olive, citrus, peach, apricot, plum, apple, pear, cherry, kiwi, almond and hazel trees.

branches come from grape-vine, fruit and olive trees pruning. On the other hand, wastes issued from vegetable and fruit processing are mainly composed by peels, leaves, seeds, and stalks.

Residues of the food farming industry can be generated by:

1. technical operations carried out on the solid material such as blurting out, peeling and removal of cores and pods, providing a waste consisting of seeds, cores, pods and stones;
2. mechanical separation and/or filtering of waste waters such as filtration of waste waters coming from preliminary washing, storage (warehousing), handling operations, giging rise to the recovery of small plant pieces and materials coming from fields;

Our attention was focused on the first class of the above described residues, representing the main residual fraction.

As for the first selection criterium, the main issue linked to the estimation of the volumes of residues coming from crop harvesting and processing was the retrieval of data related to these fluxes. As a matter of fact, only recently, institutions have conceived statistical instruments for their esteem. One of the available tools at this purpose is the MUD declaration – "Modello unico di dichiarazione ambientale"-. According to the Italian law, whoever collects and transports wastes or carries out recovering and disposal operations, including wastes treaders and middlemen, those firms and those boards that produce special non dangerous wastes coming from industrial and hand-crafted operations (art. 7, comma 3, letters c), d), g)) are obliged to declare quantities and features of the produced wastes, filling in and presenting these declarations to the autority according to Law number 25/70 of January 1994. However, these declarations were compulsory only for those farms with a business volume of 8.000.000 € (art. 2135 codice civile), and only until 2006: since then new directives exempt those farms that barely produce special non dangerous wastes from presenting any document. As a result, these declarations cannot represent a comprehensive instrument for wastes quantification, anymore.

Therefore, in order to estimate the amounts of residues produced in 2008, data reported by ISTAT on herbaceous and woody cultivations were elaborated adopting the parameter "ratio waste to product" defined by the 'Associazione Italiana di Ingegneria Agraria' (AIIA), in collaboration with Ente per le nuove tecnologie, Energia ed Ambiente (ENEA). It was thus calculated the total availability of the secondary waste (WA2: secondary waste availability) defined as the product between the total production (Ptot) and the ratio waste/product (W2/P). The category of WA2 includes all those wastes coming both from harvesting and pruning operations. Values of W2/P come from different kinds of sources, and are influenced by various factors such as culturing and harvesting techniques, plant varieties, climatic conditions, etc..

Besides, the first waste availability (WA1) was determined, corresponding to the difference between the total and the harvested production, the latter standing for the amount of products collected from soils or plants, and then moved from the production site, regardless of the final destination (definition by ANPA-ONR). It essentially refers to the amounts the products that have not been collected, thus representing a waste. The amounts of products that are not collected are strongly influenced by European legislation on allowed production rates, fixed year by year, and thus determined not simply by technical factors (e.g.: cultivation and harvesting techniques). On the contrary, these factors allow foreseeing the levels of secondary wastes.

In tables 2.1 to 2.4, data about herbaceous and woody cultures, on national and regional (Campania, South Italy) scales, are listed.
For each herbaceous and woody culture, the following parameters were defined:

- total production (Ptot), reported as tons/year
- collected product (CP), reported as tons/year
- secondary waste to product ratio (W2/P)
- percentage of humidity of the secondary waste (HW2)
- first (WA1) and secondary (WA2) waste availability, reported as tons/year

Higher values of secondary waste availability (WA2) were obtained for seasonal plants than for perennial plants. The herbaceous wastes coming across as the most abundantly produced are those obtained during the harvest of soft wheat, durum wheat and tomato on a national level; durum wheat, potato and tomato for Campania region (tab. 2.1 and 2.2). As far as woody cultures are concerned, the varieties from which the highest volume of wastes is obtained are grapevine, orange and clementine, on a national scale; peach and hazelnut in Campania (tab. 2.3 and 2.4).

	MAIN PRODUCT	SOFT WHEAT	DURUM WHEAT	MAIZE	POTATO	TOMATO	PEPPER	ARTICHOKE	WATERMELON
PARAMETERS	P	Caryopsis	Caryopsis	Caryopsis	Tuber	Berry	Berry	Bud	Fruit
	W1	Not collected product							
	W2	Straw	Straw	Stalks	Stems/ leaves	Stems/ leaves	Stems/ leaves	Stems/ leaves	Stems/leaves
	P tot t/y	3.750.336	5.187.064	9.759.473	1.798.518	4.949.846	254.674	518.000	380.869
	CP t/y	3.738.234	5.106.973	9.460.901	1.605.389	4.916.711	239.620	483.561	362.856
	W2/Ptot	0.61	0.70	n.a	0.4	0.3	n.a	n.a	n.a
	WH2 %	15	15	55	60	85	n.a	85	n.a
	WA1 t/y	12.103	80.091	298.572	193.129	33.135	15.055	34.439	18.013
	WA2 t/y	2.287.705	3.630.945	n.a	719.407	1.484.954	n.a	n.a	n.a

Table 2.1: Total availability of vegetable wastes coming from herbaceous cultivations in Italy. P: main product, W1: not collected product, W2: secondary waste, Ptot: total product, CP: collected product, W2/P: waste to total product, HW2: secondary waste humidity at its recovery, WA1: waste availability (WA1= Ptot-CP), WA2: secondary waste availability, WA2= (W2/Ptot) ∗Ptot, ha: ectars, t/y: tons per year), n.a.: data not available.

	MAIN PRODUCT	SOFT WHEAT	DURUM WHEAT	MAIZE	POTATO	TOMATO	ARTICHOKE	WATERMELON
PARAMETERS	P	Caryopsis	Caryopsis	Caryopsis	Tuber	Berry	Bud	Fruit
	W1	Not collected product						
	W2	Straw	Straw	Stalks	Stems/leaves	Stems/leaves	Stems/leaves	Stems/leaves
	P tot t/y	57,423	202,905	125,041	339,379	470,349	34,662	49,739
	CP t/y	57,239	201,147	122,815	318,784	458,933	34,180	47,800
	W2/Ptot	0.61	0.70	n.a	0.4	0.3	n.a	n.a
	WH2 %	15	15	55	60	85	85	n.a
	WA1 t/y	184	1,758	2,226	20,594	11,416	482	1,939
	WA2 t/y	35,028	142,034	n.a	135,751	141,105	n.a	n.a

Table 2.2: Total availability of vegetable wastes coming from herbaceous cultivations in Campania Region. P: main product, W1: not collected product, W2: secondary waste, Ptot: total product, CP: collected product, W2/P: waste to total product, HW2: waste humidity at its recovery, WA1: waste availability (WA1= Ptot - CP), WA2: secondary waste availability, WA2= (W2/Ptot) ∗Ptot, ha: ectars, t/y: tons per year, n.a.: data not available.

	MAIN PRODUCT		GRAPE-VINE	OLIVE	CLEMENTINE	ORANGE	PEACH	CHERRY	APPLE	PEAR	HAZELNUT
PARAMETERS	P		Berry	Drupe	Fruit	Fruit	Drupe	Drupe	False Fruit	False Fruit	Nut
	W1		Not collected product								
	W2		Shavings								
	P tot	t/y	6.584.667	3.552.090	625.971	2.287.236	1.030.992	148.080	2.224.110	923.573	146.923
	CP	t/y	6.424.969	3.471.499	451.031	2.197.295	1.012.665	134.905	2.204.744	919.953	142.109
	W2/Ptot		n.a.	n.a.	0.4	0.4	0.2	0.1	0.1	0.1	0,1
	WH2	%	50	50	40	40	40	40	40	40	40
	WA1	t/y	159.699	80.591	174.940	89.941	18.327	13.175	19.366	3.620	4.814
	WA2	t/y	n.a.	n.a.	17.388	63.534	14.319	1.028	15.445	6.413	14.692

Table 2.3: Total availability of vegetable wastes coming from woody cultivations in Italy. P: main product, W1: not collected product, W2: secondary product. Ptot: total product, CP: collected product, W2/P: waste to total product, HW2: waste humidity at its recovery, WA1: waste availability (WA1= Ptot - CP), WA2: secondary waste availability, WA2= (W2/Ptot) ∗Ptot; ha: ectars, t/y: tons per year, n.a.: data not available.

	MAIN PRODUCT		GRAPE-VINE	OLIVE	ORANGE	PEACH	APRICOT	PLUM	APPLE	PEAR	CHERRY	HAZELNUT
PARAMETERS	P		Berry	Drupe	Fruit	Drupe	Drupe	Drupe	False Fruit	False Fruit	Drupe	Nut
	W1		Not collected product									
	W2		Shavings									
	P tot	t/y	260,831	246,541	24,500	294,420	61,548	44,618	71,796	20,711	30,647	43.105
	CP	t/y	258,178	246,541	24,500	290,922	55,373	42.682	71,796	20,711	27431	42.156
	W2/Ptot		n.a.	n.a.	0.40	0.20	0.10	0.10	0.10	0.10	0.10	1.9
	WH2	%	50	50	40	40	40	40	40	40	40	40
	WA1	t/y	3,653	0	0	3,498	6,174	1,936	0	0	3,216	949
	WA2	t/y	n.a.	n.a.	9,800	58,884	6,155	4,462	7,180	2,071	3,065	103,757

Table 2.4: Total availability of vegetable wastes coming from woody cultivations in Campania Region. P: main product, W1: not collected product, W2: secondary waste, Ptot: total product, CP: collected product, W2/P: waste to total product, HW2: waste humidity at its recovery, WA1: waste availability (WA1= Ptot - CP), WA2: secondary waste availability, WA2= (W2/Ptot) ∗Ptot; ha: ectars, t/y: tons per year, n.a.: data not available.

As for food farming residues, one of the rare sources of numerical data is the 2007 annual 'Report on Wastes' drawn by APAT and ONR, on 2006 production. However, it does not report data on the fluxes of single categories of wastes (tab. 2.5).

GEOGRAPHICAL AREA	FOOD FARMING RESIDUES (t/ y)
Italy	$3*10^6$
Campania Region	$3*10^5$

Table 2.5: Food farming residues (Report on Wastes by APAT, 2007).

Numerical data on few categories of food farming wastes such as those from olive oil extraction, and from wine and tomato processing come from the website (*www.bioactive-net.com*) of the BIOACTIVE project, an European program for the valorisation of these wastes through the recovery of high added value molecules. The BIOACTIVE-NET project is a Specific Support Action (SSA) funded by the European Commission under the 6th Framework Program. The primary objective of bioactive-net is to assess and disseminate strategies for the extraction of bioactive compounds from tomato, olive, and grape processing residues to the Small and Medium Enterprises (SME) processors. As reported in BIOACTIVE-NET, according to the OIV ("International Organisation of vine and wine"), 2004 world wine production amounted to an average of 290 million hectolitres. In 2005, the French and Italian wine industries processed more than 7 million tons grapes each to produce wine with an average yield of 60-70%. During grape processing, a substantial volume of solid waste is produced, representing approximately 20% of the dry matter of the harvested grape. The pressing of 100 kg of grapes produces about 25 kg of pomace, consisting of skins (50 %), stalks (25 %) and seeds (25 %).
Another typical European cultivation is that of olives, mainly located in the Mediterranean regions. At European scale, 3 million tons olives are processed in olive oil per year (with a yield in oil of about 60.000 tons).
Olive oil production is divided into three activity fields:

- Oil mills, processing the olives into oil and producing two main byproducts such as waste water and solid waste -olive cake-.
- Plants where the oil cake is processed and residual oil is extracted from the waste resulting from olive oil extraction.
- Refineries, where the non-consumable oil is refined.

The wastes obtained after the oil milling process are mainly Olive Mill Waste Water (OMWW), alpeorujo, pomace and margine, accordingly to the applied oil extraction process.
The European olive oil milling leads to 4.5 millions m^3 of waste water per year. The 12,000 oil mills, mostly of small dimensions -some isolated, others inserted in urban contexts- have evident difficulties to unify the recovery of their wastes.
Tomato processing residues represent one of the most abundant wastes of agro-industrial sector. The total tomato production in the EU was estimated at more than 16 million metric tons in 2005 [AMITOM]. Traditionally, the most important tomato processed products are tomato concentrates: passata, puree, paste.

Of the 10 million tons of tomato processed in Europe (*http://www.bioactive-net.com*), 6 million tons were transformed in Italy (Osservatorio Agri&Food, CremonaFiere, 2008), where Campania Region (South-Italy) contributed with 500 thousand tons of processed tomatoes (*www.anicav.it*). A ratio transformed product to recovered waste of around 50 (Beni *et al*, 2006) for tomato processing gives a total amount of solid tomato residues (peels and seeds, called pomace) of about 10,000 tons in Campania region.
Another food farming residue which has been taken into account is that from apples processing. Apples constitute one of the main Italian cultures, with 2 million tons produced in 2006 at national scale, bringing Italy among the main apples producer countries in Europe and at the fifth place on world scale. Besides, Campania region stands out with around 70,000 t of apples produced in 2006 (Istat, Cultivations, 2008). 5 % of collected fruits is transformed, giving 100,000 t apples processed in Italy, and 3,600 tons in Campania, getting to 2,000 t and 70 t of solid wastes produced in Italy and in Campania, respectively.
In Italy, as well as in Campania other varieties of fruit are processed, such as orange, lemon, peach and apricot, even if quantitative data are not available for the resulting wastes (peelings, seeds, and stalks).
Other classes of special non dangerous wastes that have been taken into account are the organic and green fractions of municipal solid wastes, the latter essentially consisting of branches coming form wood shaving. Table 6 quotes the amounts of these waste fractions related to Italy and Campania Region. The potential of exploting organic fraction is clear if one takes into account the volumes yearly produced on a national and regional scale. In Campania Region, for example, in 2005 almost 90,000 t of organic fraction selected by citizens were produced. If one conceives to use these fractions as raw materials for bioethanol production, the inherent advantage is related to the huge volumes that compensate for the lower yield of alcohol obtainable from this class of substrate (tab. 2.7).

AREA	ORGANIC FRACTION	GREEN FRACTION (1)
	(t/ y)	
Italy	$2*10^6$	$1*10^6$
Campania	$9*10^4$	$3*10^4$

Table 2.6: Data about organic fraction and green fraction of urban solid wastes, from Report on Wastes by APAT, 2007. (1): Pruning residues of urban green, etc..

RAW MATERIALS	Yield
	(l/ t)
Apples	61
Municipal solid wastes	68
Potatoes	96
Tree shavings	176
Other agrarian residues	189
Wheat stalks	227
Forestry residues	250
Cellulose	259

Table 2.7: Theorical yields of bioethanol obtainable from different ligno-cellulosic materials.

2.1.2.2 Periodicity of their production

Furthermore, the periodicity of waste production was considered, since a continuous supply of raw materials for the following operations is required to set up a continuous and economically advantageous process.

Data about the time of harvest of fruits and vegetables, hence of production of their relative wastes, have been deduced from the 2001 APAT/ONR Report on Special Wastes (tab. 2.8). Besides, data about periodicity of production of food farming residues, reported in table 2.9, were obtained from direct interviews gathered by EURECO s.p.a. with different companies located in Campania Region (La Doria, ditta Coppola, ditta Mancuso, la Colombaia).

From the analysis of available data, it turns out clear that both the production and availability of fruit and vegetables, and of their processing wastes are restricted to a short period of time. Given the short times of wastes production it's hard to think of a process based only on a single category of waste, although easier to manage. The target should be to conceive a flexible process in which the exploitation of a panel of lignocellulosic residues is accomplished.

As far as the last category of wastes is concerned, organic and green fractions of solid municipal wastes have as main advantage the fact of being continuously produced all along the year.

PRODUCTS	WASTE	TIME OF PRODUCTION
Cereals	Straw	June-July
Rice	Straw	End of October
Maize	Stalks/ Caryopses	October-November
Sunflower	Stems/ Leaves/ Seeds	August-September
Tomato	Leaves/ Stems Seeds/Peelings	End of July-September
Potato	Tubers/ Leaves/ Stems	Winter-Spring cultivation: March-June
		Spring-Summer cultivation
Artichoke	Leaves/ Stems/Buds	June-October
Citrus	Shavings/ Fruit	Each two years, beginning of Spring
Peach/ Apricot	Drupes/ Shavings	June
Apple/ Pear	False fruits/ shavings	Summer-November

Table 2.8: Time of production of disparate agrarian products and of their residues (APAT and ONR Report, 2001).

WASTE	TIME OF PRODUCTION	DATA SOURCES
Tomato	End of July-September	La Doria
Pickles- residues of celery, peppers, carrots, fennels, onions, turnip	Depending of vegetable time of production	Mancuso (Saclà)
Fruit semi-finished goods: apricot, peaches, apples, pears	Half of June and till the end of Novembre	Coppola

Table 2.9: Time of production of food farming wastes (Data from direct interviews carried out by EURECO Environmental Company S.p.a.).

2.1.2.3 Common ways for lignocellulosic wastes disposal or reuse

Following the General EU Legislation on wastes (Directive 2006/12/EC), Member States shall take the necessary measures to ensure that wastes are recovered or disposed of without endangering human health and without using processes or methods which could harm the environment.

The residues coming from herbaceous and woody cultures are generally considered as coproducts and even after their zootechnical employment (as animal fodder or as litter components), they usually return to the ground, being mostly exploited for humus production, building a brief circuit way of recycling the organic substance.

Also in the case of fruit and vegetables processings, the resulting wastes are used as fertilizer and as animal fodder, even if this is mainly the case for the most valuable ones, such as tomato pomace, maize and fruit residues. Data on fruit transformation residues reported by Rossi from 'Centro Ricerche per le Produzioni animali' (CRPA) (2006) show that the commonest way for their disposal is distillation, for the production of alcoholic beverages.

The byproducts obtained during tomato transformation process are defined as Secondary Raw Materials. Council Directive 96/25/EC legislates the re-use in particular of "tomato pulp obtained by pressing *Solanum lycopersicum* Karst. tomatoes during the production of tomato juice" for animal feeding. The pomace is currently sold, transferred to other companies without any monetary exchange or removed at the expense of tomato processors.

The wineries in the South European countries are obliged to bring their wastes to the distilleries according to EC Regulation 1493/99, where the common forms of further processing are the recovery of spirits via distillation and extraction of grape seed oil, followed by incineration of the final solid waste, as it happens to other fruit processing wastes. However, currently, the grape processing residues are disposed of spreading them on the land, using them as animal fodder, burning, landfilling or using as biomass or compost. However, the trade value of dregs of grapes depends on humidity, grape stalks presence and amount of sugars and alcohol.

The organic and green fractions of solid urban residues have as main application the production of compost, used as fertilizer. The quality of compost varies according to the quality of the original materials, and different categories of composted materials can then be obtained. Transforming organic and green fractions of municipal wastes into a resource could represent a turning point, if one takes into account that Campania has been experiencing an emergency state about the collection and disposal of municipal solid wastes for more than a decade. Furthermore, Campania administration used to export even the most valuable wastes, such as organic fraction, to other regions in Italy or in Europe (mainly Germany). A possible solution could be to employ them for high added value products recovery, thus not only gaining environmental and economical advantages, but also inducing people towards the separate collection of rubbish. In this way, a virtuous circle would be triggered, increasing amounts of separately collected wastes, employing them for the extraction of valuable molecules and for biofuel production, and thus reducing environmental impact and the total cost of garbage management.

2.1.2.4 Macromolecular composition and its variability

It is difficult to find data on the macromolecular composition of agro-industrial wastes. Available data on macromolecular composition of agrarian and food-farming residues have been found mainly on websites and taken from literature, as summarised in table 2.10. Some data about agrarian residues are available in the *Phyllis* database

of the Energy Research Centre of the Netherlands –ECN- (*http://www.ecn.nl/phyllis/*). It is reported that they are mainly composed of cellulose (32%) and starch (38%), with lower levels of hemicellulose (17%). *Phyllis* database also reports data on food farming residues coming from potato processing (tab 2.10). This waste, essentially composed of peels, can have a variable content both of cellulose and starch, and low levels of hemicellulose. Orange peelings present similar levels of cellulose, pectin and hemicellulose, and lower content of lignin (Mamma *et al.* 2007). Mandarin peelings, according to Osma *et al*. (2007), have lower levels of cellulose and lignin than the other considered residues. As reported by Botella *et al*. (2005), grape pomace can be divided into two main fractions, one mainly composed of seeds, and the other of peelings. The first one has high lignin content (64%) and a lower cellulose and hemicellulose content (18%). The second fraction has lower levels of cellulose, and similar contents of hemicellulose and lignin (31%). Wolter *et al*. (1980) determined the fibre content of apple pomace, showing the presence of similar amounts of cellulose (23%) and lignin (19%), and low levels of hemicellulose (6%). Apples wastes show high potential for their upgrading, considering their composition and the presence of compounds with good market possible shares. Therefore they have been subjected to numerous investigations. Apple pomace has been described as a rich source of polyphenols such as cinnamic acids (caffeic acid), cinnamic acid derivatives (p-coumaric glucoside, p-coumaroylquinic and chlorogenic acids), flavonols (hyperoside, isoquercitrin, reynoutrin, avicularin, quercitrin, quercetin), flavan-3-ols (catechin, epicatechin and procyanidins). These molecules make it interesting to set up a process in which they are recovered and from the resulting residues the polysaccharides and all the other nutrients present inside are exploited (Sanchez-Rabaneda e*t al.*, 2004).

As far as tomato pomace composition is concerned, Del Valle *et al*. (2006) showed that it has a high protein content, low fat and pectin content, and interesting levels of total sugars.

The high variability of composition of organic fraction of municipal solid wastes, in terms of the present materials, influenced by season and place of production (different regions), is the main drawback associated with the exploitation of this class of residues.

WASTES	PROTEIN	TOTAL SUGARS	REDUCING SUGARS	CELLULOSE	PECTIN	HEMICELLULOSE	LIGNIN	STARCH	REFERENCES
				(%)					
Agrarian residues	n.r.	n.r.	n.r.	32	n.r.	17	n.r.	38	*Database* Phillis, Energy centre of the Netherlands
Potato processing residues	n.r.	n.r.	n.r.	10-26	n.r.	11.8	n.r.	40-70	
Orange peelings	7.9	n.r.	n.r.	16.2	14.4	13.8	1.0	n.r.	Mamma *et al*., 2007
Mandarin peelings	n.r.	n.r.	n.r.	12-14	n.r.	n.r.	9-11	n.r.	Osma *et al*., 2007
Orange bagasse	n.r.	19	9	n.r.	0.1	n.r.	n.r.	n.r.	Martin *et al*., 2004
Sugar cane bagasse	n.r.	10.4	3.5	n.r.	n.r.	n.r.	n.r.	n.r.	
Wheat bran	n.r.	16.7	5.2	n.r.	n.r.	n.r.	n.r.	n.r.	
Grape pomace/ seeds	9.3	8	7.1	17.7	0.2	18	64	n.r.	Botella *et al*., 2005
Grape pomace/ peelings		13		6	4	31	31	n.r.	
Apple pomace	n.r.	n.r.	n.r.	22.9	n.r.	6.2	19.1	17.9	Wolter *et al*., 1980
Tomato pomace	19.3	25.7	n.r.	n.r.	7.5	n.r.	n.r.	0	Del Valle *et al*. 2006

Table 2.10: Percentual composition of agrarian and food farming residues (%: percentage of the dry matter); n.r.: data not reported).

2.1.3 Conclusions

In order to select lignocellulosic waste materials to be further exploited in an economical and evironmental friendly process bringing to several high added value products, data have been gathered on those wastes coming from herbaceous and woody cultures and from food processing processes strongly linked to the territory. Besides, the possibility of employing the green and organic fractions of municipal solid wastes has been considered.

Taking into account only data on discharged volumes, residues from herbaceous and woody cultivations (tables 2.1- 2.4) are of main interest, and the following wastes were chosen as the most representative:

Herbaceous cultures:

- soft wheat, durum wheat and tomato, in Italy;
- potato, tomato and durum wheat, in Campania;

Woody cultures:

- grape-vine, clementine, orange, in Italy;
- peach and hazelnut, in Campania.

The use of these wastes has as the main drawback the fact that they are usually left on the ground, to produce humus: process of crucial importance for the maintenance of soil fertility. This consideration has led us to exclude the exploitation of these wastes, being the focus of the project to employ materials not having a way for their re-use yet or to create a process adding a higher value to the selected wastes. Besides, the costs linked to their recovery would be too high to justify their use, eliminating the economical advantage of using wastes as raw materials, without setting up an efficient system for their collection and with the transformation plant far from the production site. As far as food farming residues are concerned, the presence of farms with the problem of wastes disposal highlights the importance of finding a way for their exploitation, as an alternative to landfilling, burning, animal feeding. Remarks about advantages and disadvantages on the use of different waste materials are listed in table 2.11.

Thus, **tomato and apple pomaces** were selected as starting raw materials.

Tomato residues were chosen because of the high amounts yearly produced, and the need of tomato processing industries to find alternative ways for their disposal. On the other hand, apple pomace appeared to be exploitable mainly for its composition and presence of valuable molecules with high market profitability. If the time of food farming wastes production is taken into account (fig. 2.1), the idea could be to exploit a panel of different wastes in a single process. If waste production times partially overlap, an almost continuous supply of materials can be ensured for the following operations. In order to keep the continuity of substrates supply, an alternative strategy could be the use of organic fraction of municipal wastes or of wood shaving residues, produced all year long. This choice, however, implies the use of materials with the macromolecular composition changing accordingly to season and geographical origin. To fully exploit these fractions, it would be amenable to develop a flexible process, adaptable to different materials aimed at their upgrading by means of the obtainment of high added value products such as sugars, valuable molecules (e.g.: colorants, antioxidants) and industrially relevant enzymes.

WASTES	ADVANTAGES	DRAWBACKS
AGRARIAN RESIDUES	High volumetric production High polysaccharides content	Difficulties for their recovery Employed for humus production
FOOD FARMING RESIDUES	Compositional homogeneity Availability of local farms to furnish them Strong connection with the territory Absence of other alternative uses	No availability of quantitative data
FRUIT AND VEGETABLE RESIDUES/ ORGANIC FRACTION OF MUNICIPALE SOLID WASTES	High volumetric production Continuity of production	Not even macromolecular composition

Table 2.11: Advantages and drawbacks of the exploitation of disparate classes of special not dangerous wastes.

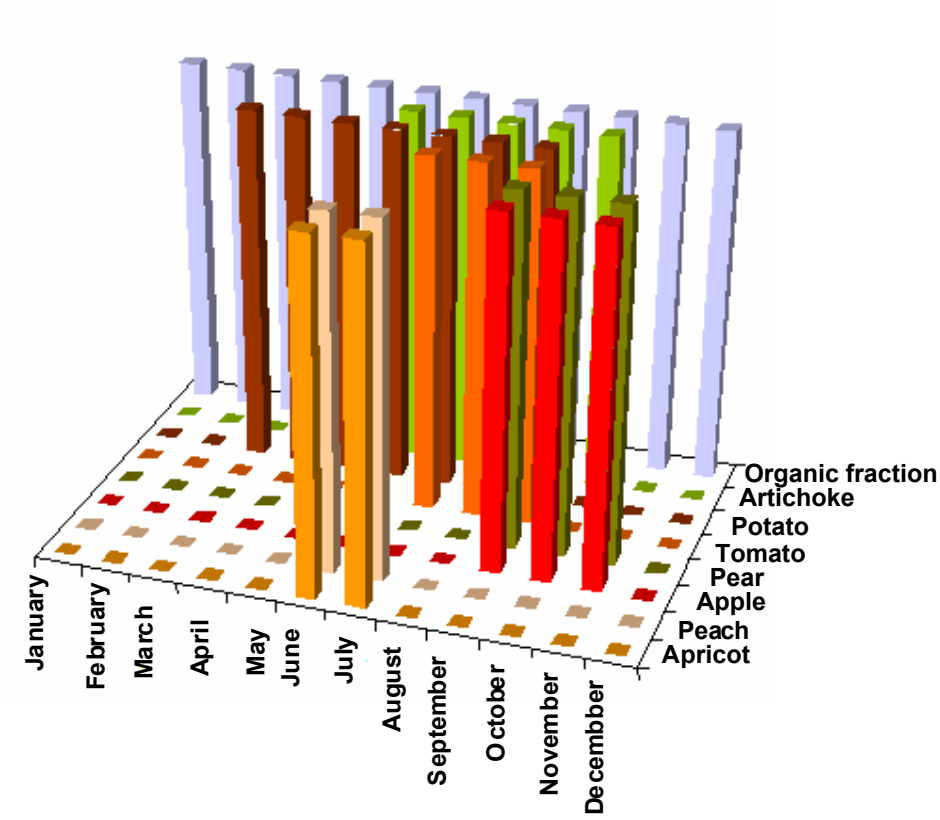

Figure 2.1: Times of production of wastes coming from agrarian cultivations, food farming processes and of the organic fraction of urban wastes (data collected by Eureco environmental group s.r.l., and taken from the APAT e ONR Report on special wastes in 2001).
Different kinds of wastes, coming from: Apricot: ■; Peach: ■; Apple: ■; Pear: ■; Tomato: ■; Potato: ■; Artichoke: ■; and Organic fraction of urban solid residues: ■.

2.1.4 References

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2.2. Selection of lignocellulolytic fungi

2.2.1 Introduction

The complexity of the ligno-cellulose matrix imposes a huge work in order to find effective treatment practices allowing its exploitation as starting raw material of biorefinery. A further goal of this work was, therefore, to select micro-organisms to be employed in the conversion of the different macromolecular components (lignin, pectin, cellulose and hemicellulose) present in the selected ligno-cellulosic wastes (section 2.2). Fungi have been chosen as conversion microorganisms given their natural ability to colonize lignocellulosic materials, such as wood. As a matter of fact, these micro-organisms produce different classes of hydrolytic (i.e. cellulases, hemicellulases, pectinases) and oxidative (i.e. ligninases) enzymes, taking part in the conversion of the different macromolecular components present in their lignocellulosic substrates.

In lignocellulose, lignin and, when present, pectin constitute a physical barrier, surrounding cellulose and hemicellulose, and hindering the access of the hydrolytic enzymes to the polysaccharides, thus preventing their hydrolysis into monosaccharides. Huge efforts have been focusing on improving yield of lignocellulose conversion, since the produced sugars can be further processed producing biofuels, such as ethanol and butanol, solvents and other chemicals, as a result of yeast or bacterial fermentation.

Fungal enzymes involved in lignin degradation belong to the class of oxidases, such as laccases and veratryl alcohol oxidases, and peroxidases, such as manganese and lignin peroxidases (MnP and LiP, respectively) (Hatakka, 1994). On the other hand, pectin is hydrolised by pectinases and polygalacturonases (Phutela ***et al.***, 2005). After lignin and pectin removal, sequential hemicellulose and cellulose conversions take place. In the former process, several hydrolytic activities are involved, such as endo-β-1,4-xylanase, exo-β-1,4-xylanase, endo-acting β-mannanases, exo-acting β-mannosidase, and other auxiliaries ones such as α-L-arabinofuranosidase, acetylxylan esterase (Polizeli ***et al***., 2005). On the other hand, for cellulose complete saccharification at least three different activities have to be present (Baldrian ***et al***., 2008). Two of them act either on the backbone (endoglucanases), or on the extremities of the polysaccharide (exo-glucanases). The sinergistic action of these enzymes produces cellobiose, that is in turn hydrolysed by the third class of enzymes, the β-glucosidases.

In order to select fungi potentially active in lignocellulose conversion, data from literature were collected on fungal performances during solid state fermentation experiments on lignocellulosic waste materials.

2.2.2 Results

Results are reported in terms either of changes in the macromolecular composition due to fungal growth or of levels of lignino-cellulolytic activities produced during the process. Besides, many manuscripts report the utilization of agrarian and food farming residues as support and source of nutrients, with or without nutrients addition.

The available data are summarized in the tables from 2.12 to 2.18, according to the following criteria:

1. changes in macromolecular composition (tab. 2.12-2.14);
2. produced conversion enzymatic activities (tab. 2.15-2.18).

In the tables, the different substrates adopted in the considered manuscripts are specified.
As far as the first kind of classification is concerned, *Trametes versicolor* NBRC (IFO) 4937 and *Phanerochaete chrysosporium* NBRC (IFO) 31249 stand out, being characterised by an almost equivalent and significant degradation ability towards both lignin and cellulose components, when grown on rice straw. However, *T. versicolor* determines a more significant reduction in both macromolecular components (giving 58 and 37% of cellulose and lignin degradation, respectively) than *P. chrysosporium* (reducing the cellulose and lignin contents by 49 and 21%, respectively) (tab. 2.12). *Pleurotus tuber-regium* shows lignin and hemicellulose conversion ability when grown on wheat straw. Whereas, *P. ostreatus* ATCC 66376 is able to transform both cellulose and lignin with the same efficiency, when grown on rice.

Strain	Substrate	Dry weight loss	Hemicellulose loss	Cellulose loss	Lignin loss	References
		(%)				
Trametes versicolor NBRC(IFO) 4937	Rice straw	n.a.	n.a.	58	37	Taniguchi *et al*., 2005
Phanerochaete chrysosporium NBRC(IFO) 31249		n.a.	n.a.	49	21	
Ceriporiopsis subvermispora ATCC 90467		n.a.	n.a.	n.a.	18	
P. ostreatus ATCC 66376		n.a.	n.a.	32	30	
P. tuber-regium PT1	Wheat straw	31.5	72.8	34.3	61.7	Jalc *et al*.,1999
P. tuber-regium PT4		20.9	82.1	18.5	70.7	
P. tuber-regium PT5		4.8	54.9	2.1	31.3	
Xylobolus frustulatus	Populus tremuloides	26	n.a.	n.a.	n.a.	Otjen *et al*., 1985
Ischnoderma resinosum		22	n.a.	n.a.	n.a.	
Poria medulla-panis		15	n.a.	n.a.	n. a.	
Lentinula edodes	Beech litter	57.6	n.a.	n.a.	59.8	Osono *et al*., 2002
Microporus vernicipes		15.1	n.a.	n.a.	6.5	
Mycena polygramma		36.6	n.a.	n.a.	33.2	
Naematoloma sublateritium		23.9	n.a.	n.a.	16.3	
Xylaria carpophila		14.4	n.a.	n.a.	3.9	

Table 2.12: Percentual changes in the macromolecular composition of ligno-cellulosic materials due to fungal colonization. (n.a.: not available)

Table 2.13 lists the fungi that have been isolated from decaying wood. They were shown able to selectively degrade lignin, determining substantial losses (86 and 99%) in lignin content of the colonized substrates.

In the table 2.14, changes in oat straw and alfa-alfa grass composition are reported, due to the colonization by the fungi *P. chrysosporium*, *Pholiota mutabilis*, *Phellinus pini*, *Phlebia tremellosa* and *Scytinostroma galacticum*. All fungi determined no significant change in hemicellulose content on both substrates. *P. chrysosporium*, however, determined a slight reduction both in cellulose and in lignin content, when grown on oat straw. Both this fungus and *P. tremellosa* were active on cellulose component of alfa-alfa grass, lowering its content by 26 and 37 %, respectively.

Strain	Substrate	Lignin Loss (%)	References
Bjerkandera adusta	Birch	87.7	Blanchette, 1984
Cerrena unicolor		90.9	
Perenniporia medulla-panis		86.2	
Ganoderma tsugae	Hemlock	95.8	
Inonotus dryophilus	Oak	91.7	
Perenniporia subacida	Fir	95	
Phellinus pini		99	
Heterobasidion annosum		92	

Table 2.13: Changes in lignin content of four different wood species due to fungal growth

Strain	Substrate	NDF	Hemicellulose	Cellulose	Lignin	References
		(% d.m.)				
Control	Oat straw	88.2	26.9	50.5	10.2	Jung, 1992
P. chrysosporium		68.1	21.0	38.4	6.6	
Pholiota mutabilis		87.7	26.0	52.3	9.7	
Phellinus pini		86.0	25.1	50.2	10.1	
Phlebia tremellosa		78.4	22.2	46.9	8.5	
Scytinostroma galacticum		86.5	26.8	50.1	10.9	
Control	Alfa-alfa	68.4	15.9	40.9	11.5	
P. chrysosporium		61.2	17.0	30.1	14.2	
P. mutabilis		72.7	15.2	43.9	13.7	
P. pini		68.8	14.6	40.9	13.3	
P. tremellosa		65.6	16.5	25.6	17.3	
S. galacticum		65.5	17.0	33.2	15.2	

Table 2.14: Composition of neutral detergent fibre of oat straw and alfa-alfa after 30 days of fungal growth; NDF: neutral detergent fibre made up by cellulose, hemicellulose, lignin, cutin.

As far as enzymatic activity levels are concerned, most authors report low levels of laccase activities with different fungi on different substrates (tab. 2.15). Both Moldes (2003) and Rosales (2004) obtained high levels of laccases with *Trametes hirsuta* both on grape seeds (69 U/g) and kiwi peelings (32 U/g), respectively (tab. 2.15). It is worth noticing that *P. ostreatus* secretes varable amounts of laccases depending on the adopted substrate. However, in all the investigated consitions, laccase levels produced by *P. ostreatus* were lower than those secreted by most of the other reported microorganisms. Low levels of MnP were found for every reported fungi in the explored conditions, with *Cerrena unicolor* secreting the highest amount (3.8U/g) on fibre and de-inking sludge (tab. 2.15).

Strain	Substrate	Laccase (U/g)	MnP (U/g)	References
P. ostreatus	Wheat straw	1.36	0.79	Valaskova *et al.*, 2006
T. versicolor		n	0.26	
P. betulinus		n.d.	n.d.	
T. pubescens	Mandarin peelings	7.77	n.a.	Osma *et al.*, 2007a*
	Glucose	5.10	n.a.	
	Glycerol	8.90	n.a.	
L. edodes IBB123	Tree leaves	14.2	1.67	Elisashvili *et al.*, 2008*
L. edodes IBB363		13	0.25	
L. edodes IBB369		1.7	1.15	
P. drynus IBB903		4	1.42	
P. ostreatus IBB8		1.7	2	
P. ostreatus IBB108		3.5	1.57	
P. ostreatus 2175		3.7	1.87	
P. ostreatus 2191		3.5	1.97	
P. tuberregium IBB624		5	0.52	
L. edodes IBB123	Wheat straw	5	1.3	
L. edodes IBB363		13.7	1.22	
L. edodes IBB369		9.5	0.9	
P. drynus IBB903		3.2	1.42	
P. ostreatus IBB8		1.7	1.47	
P. ostreatus IBB108		2.5	0.27	
P. ostreatus 2175		3	1.87	
P. ostreatus 2191		4.2	2.52	
P. tuberregium IBB624		2.5	0.55	
Trametes versicolor	Barley bran	4.8	n.d.	Rodriguez Couto *et al.,* 2002*
T. hirsuta	Grape seeds	69	n.d.	Moldes *et al*., 2003*
	Potato peelings	7.5	n.d.	Rosales *et al*., 2002*
	Groundnut shells	0.3	n.d.	Rodriguez Couto *et al*., 2006*
	Kiwi peelings	32	n.d.	Rosales *et al*., 2004*
T. pubescens CBS 696.94	Banana waste	4.5	n.d.	Osma *et al*., 2007b*
	Mandarin peelings	2.5	n.d.	Osma *et al*., 2007a*
Cerrena unicolor T71	Oat husks FDS	10.6	3.8	Winquist *et al*., 2008*

Table 2.15: Lignino-cellulolytic enzymes produced by *Pleurotus ostreatus* CBAS477, *Trametes versicolor* CCBAS614, *Piptoporus betulinus* CBAS585, and *Trametes pubescens* and several species of *Lentinus edodes and of Pleurotus* during solid state fermentation experiments. *: in these experiments a synthetic medium was added. MnP: Manganese peroxidase; n: negligible: lower than 0.1 U/g; n.d.: not detected; n.a.: not available, FDS: fibre and de-inking sludge.

From tables 2.16 to 2.18, levels of glycosyl hydrolases are reported. Considerable values of polygalacturonases were achieved with different strains of the genus *Aspergillus*, such as *Aspegillus fumigatus* Fres. MTCC 4163 (TF3), *Aspergillus* spEG66F and *Aspergillus* spEGC4, grown on pectin, sugar cane bagasse or wheat bran (642, 894 and 1,518 U/g, respectively) (tab. 2.17).
Moniliella spSB9 and *Penicillium* spEGC5 produce high levels of polygalacturonase activity when grown on mixtures of wheat bran to sugarcane bagasse at different ratios (19,000 and 9,000 U/g, respectively). These strains also secrete huge amounts of pectinases when grown on sugarcane bagasse, wheat bran and orange bagasse mixed in equal amounts. Very high levels of pectinases were also obtained with the

fungus *Penicillium viridicatum* RFC3 on different substrates such as wheat bran, maize teguments and orange bagasse.
The fungus *Neurospora crassa* DSM1129 was reported to produce high levels of endo-glucanase activity when cultivated on Water Unextractable Orange Peels (WUOP) (138.5 U/g). The strains 2175 and 2191 of *P. ostreatus* and *P. drynus* IBB903 were good producers of endoglucanases on tree leaves, with the latter fungus showing noteworthy performances also on wheat straw (tab. 2.18). *P. drynus* IBB903 and *P. ostreatus* 2175 secrete the highest amounts of Filter paper activity (FPA, as a measure of exo- and endo acting glucanases), among reported fungi. *P. drynus* IBB903 and the two strains 2175 and 2191 of *P. ostreatus* were the best producers of xylanase activity, both on tree leaves and wheat straw. Strains 2175 and 2191 of *P. ostreatus* were both good producers of the above reported enzymatic activites, in the same growth conditions, even if *P.ostreatus* 2191 was the best on wheat straw.

Strain	Substrate	1,4-β-xylosidase	1,4-β-mannosidase	Endo-1,4-β-xylanase	1,4-β-glucosidase	Endo-1,4-β-glucanase	Cellobiohydrolase	References
		(U/g)						
P. ostreatus	Wheat straw	n	n	n.d.	n	n	n	Valaskova *et al*., 2006
T. versicolor		n	n	n	0.36	0.43	0.10	
P. betulinus		0.26	0.59	1.92	1.42	20.06	0.15	
T. pubescens	Mandarin peelings	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Osma *et al*., 2007a
	Glucose	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	
	Glycerol	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	

Table 2.16: Extractable glycosil hydrolases by *Pleurotus ostreatus* CBAS477, *Trametes versicolor* CCBAS614, *Piptoporus betulinus* CBAS585, and *Trametes pubescens* solid state fermentation experiments (n: negligible: lower than 0.1 U/g; n.d.: not detected; n.a.: not available).

Strain	Substrate	Poly-galacturonase	Pectinase	endo-1,4-β-glucanase	Xylanase	References
		(U/g)				
Aspergillus awamori	Wine grape pomace	25	n.a.	n.a.	40.4	Botella *et al*., 2006
A. fumigatus Fres. MTCC 4163 (TF3)	Pure Pectin	642	569	n.a.	n.a.	Phutela *et al*, 2005
	Citrus sinensis	283	264	n.a.	n.a.	
	Malt sprouts	455	452	n.a.	n.a.	
	Rice bran	489	452	n.a.	n.a.	
	Pomegranate	474	437	n.a.	n.a.	
	Orange	299	636	n.a.	n.a.	
	Lemon	401	408	n.a.	n.a.	
	Banana	387	361	n.a.	n.a.	
Penicillium vidiricatum RFC3	Orange bagasse	12	2,000	n.a.	n.a.	Silva *et al*., 2002
A. fumigatus (TF3)	Wheat bran	625	589	n.a.	n.a.	Phutela *et al*, 2005
P. viridicatum RFC3		30	1,200	n.a.	n.a.	Silva *et al*., 2002

Table 2.17: Enzymatic activities produced during solid state fermentation experiments on agrarian and food farming residues (n.a.: data not available; WUOP: *water unextractable orange peels*.

Strain	Substrate	Poly-galacturonase	Pectinase	Endo-1,4-β-Glucanase	β-Xylosidase	References
		(U/g)				
A. niger BTL	WUOP	135.7	130.8	60.5	77.1	Mamma *et al.,* 2008
Fusarium oxysporum F3		91.4	38	69.5	28.9	
Neurospora crassa DSM1129		63.3	29.1	138.5	56.8	
P. decumbens		56.3	49.6	45.5	37.8	
Aspergillus spEG66F	90% Sugar cane bagasse 10% wheat bran	894	10.2	n.a.	n.a.	Martin *et al.,* 2004
Aspergillus spEGC4		330	2.4	n.a.	n.a.	
Curvularia inaequalis EM P11-1		372	8	n.a.	n.a.	
Moniliella spSB9		16,800	3.2	n.a.	n.a.	
P. citrinum		602	7.8	n.a.	n.a.	
P. glabum F1		51	14.4	n.a.	n.a.	
P. itallicum		876	16	n.a.	n.a.	
*P. sp*EGC5		2,540	8.7	n.a.	n.a.	
P. spRFC2		1,002	7.2	n.a.	n.a.	
Thermoascus sp179.5		368	27	n.a.	n.a.	
Moniliella spBB9	Orange bagasse: wheat bran: sugar cane bagasse (1: 1: 1)	26	19,400	n.a.	15.5	
Penicillium spEGC5		12	11,000	n.a.	12	

Table 2.17: Continued

Strain	Substrate	Poly-galacturonase	Pectinase	Endo-1,4-β-Glucanase	β-Xylosidase	References
		(U/g)				
Aspergillus spEGC4	Wheat bran 90% sugar cane bagasse 10%	1,518	11.4	n.a.	n.a.	Martin *et al.,* 2004
Aureobasidium spRE		81	9	n.a.	n.a.	
Aspergillus spEG66F		83	15	n.a.	n.a.	
Cladosporium spRFC1		56	11	n.a.	n.a.	
C. inaequalis EM P11-1		n.a.	15	n.a.	n.a.	
Moniliella spSB9		18,740	21	n.a.	n.a.	
P. itallicum		n.a.	16	n.a.	n.a.	
P. spEGC5		9,250	11	n.a.	n.a.	
P. spRFC2		n.a.	10.2	n.a.	n.a.	
P. viridicatum RFC3		656	19	n.a.	n.a.	
Phanerochaetes sp291		720	21	n.a.	n.a.	
Thermoascus sp179.5		222	34	n.a.	n.a.	
P. v. RFC3	Sugar cane bagasse + wheat bran	27	1,500	n.a.	n.a.	Silva *et al*., 2002
	Sugar cane bagasse + orange bagasse	9	2,500	n.a.	n.a.	
	Sugar cane bagasse + mango peelings	15	1,500	n.a.	n.a.	
	Sugar cane bagasse + maize teguments	5	500	n.a.	n.a.	
	Sugar cane bagasse + banana peelings	15	1,000	n.a.	n.a.	
	Orange bagasse + wheat bran	55.2	3,540	n.a.	n.a.	

Table 2.17: Continued

Strain	Substrate	Endo-1,4-β-Glucanase	Xylanase	FPA	References
		(U/g)			
L. edodes IBB123	Tree leaves	10	50	11.2	Elisashvili *et al.*, 2008
L. edodes IBB363		16.25	38.7	11.2	
L. edodes IBB369		12.5	21.2	10	
P. drynus IBB903		282.5	536	51.2	
P. ostreatus IBB8		6.2	40	10	
P. ostreatus IBB108		32.5	55	13.7	
P. ostreatus 2175		300	546.2	56.2	
P. ostreatus 2191		325	360	26.2	
P. tuberregium IBB624		37.5	38.7	21.2	
L. edodes IBB123	Wheat straw	86.2	68.5	15	
L. edodes IBB363		33.7	49.7	11.2	
L. edodes IBB369		45	63.7	12.2	
P. drynus IBB903		227.5	362.5	30	
P. ostreatus IBB8		46.2	65	5	
P. ostreatus IBB108		61.2	65	15	
P. ostreatus 2175		60	161.2	22.5	
P. ostreatus 2191		165	183.7	20	
P. tuberregium IBB624		37.5	65	16.2	

Table 2.18: Cellulolytic enzymes produced by *Lentinus edodes* and several species of *Pleurotus* during SSF experiments on tree leaves and wheat straw (CM-Case: Carboxymethyl cellulases: endoglucanase activity, FPA: Filter paper activity, total cellulase activity).

2.2.3 Conclusions

A comprehensive result of this work was setting up an internal data bank of fungi selected according to their conversion abilities towards the different macromolecules composing lignocellulosic materials. At this purpose, the above reported data on compositional changes and on secreted enzymatic activities levels were taken into account.

As far as the conversion performances are concerned, *T. versicolor* NBRC (IFO) 4937 stands out as both cellulolytic and ligninolytic fungus, *P. tuber-regium* can be selected as hemicellulose converter whilst *Phellinus pini* results the best lignin degrader.

The panel of selected microorganisms was further enlarged, taking into account the enzymatic activities involved in lignocellulose conversion. *T. hirsuta* stands out as producer of laccase activity (Moldes, 2003; Rosales, 2004). *Moniliella* spSB9 produces huge amounts of polygalacturonases and pectinases, thus resulting the best fungus for transforming pectin. *P. ostreatus* 2191 appears the best endoglucanase producer, while *P. ostreatus* 2175 produces the highest levels both of xylanase and filter paper activities. *Aspergillus niger* BTL and *Neurospora crassa* DSM1129, intriguingly, secrete high amounts of different enzymes such as polygalacturonases, pectinases, endoglucanases and xylanases, thus resulting useful for the conversion of different polysaccharidic fractions at the same time. As well, *P. dryinus* IBB903, secreting high amounts of different classes of glycosyl hydrolytic enzymes, could be used for the concomitant conversion of polysaccharidic components of ligno-cellulose. It is worth noting that fungal performances in lignocellulose conversion are strongly influenced by the exploited substrate as shown by analysed data. Thus, it seems of high relevance the high methabolic adaptability shown by fungi belonging to *Pleurotus* genus, as reported in the examined papers.

For the development of solid state fermentation on apple and tomato processing residues, main objective of this thesis, *P. ostreatus* and *T. versicolor* were chosen as process microorganisms (Fig. 2.2).
P. ostreatus was selected considering its ability of producing xylanase and cellulase activities, as above described. A strain -(Jacq.:Fr.) Kummer (type: Florida) ATCC no. MYA-2306- of this fungus has been deeply studied in the laboratory where this research project has been carried out. The phisiology of laccases production and secretion in liquid cultures has been characterised, discovering that the fungus secrets multiple isoforms of this enzyme, depending on the stage of development and the environmental conditions (Palmieri *et al*., 2000; Palmieri *et al*., 2003). On the other hand, *P.ostreatus* genome annotation has been recently accomplished (*http://genome.jgi-psf.org/PleosPC15_1*), thus strengthening the instruments for its exploitation and for further physiological studies and biotechnological applications. Our interest in exploiting *P. ostreatus* lignocellulose conversion by solid state fermentation processes was confirmed by the above reported analysis of literature data, showing that this fungus is a good lignocellulose convertor. As a matter of fact, it has very good performances in the conversion of lignin, thanks to the secretion of oxidative enzymes, but is also able to modify the polysaccharides present in the substrate.
The other fungus that was selected is *T. versicolor* NBRC(IFO) 4937, being able of both lignin and cellulose conversion as shown by examined literature data. *Trametes (Coriolus, Polyporus) versicolor* is a lignin-degrading basidiomycete (Eriksson *et al.*, 1990), secreting oxidases (laccases) and peroxidases (MnP and LiP). This strain was shown to secrete two laccase isoforms, in different ratios depending on culture conditions such as presence of laccase inducers (Bourbonnais *et al*., 1995) and exploited lignocellulosic substrate (Moldes *et al*., 2004).

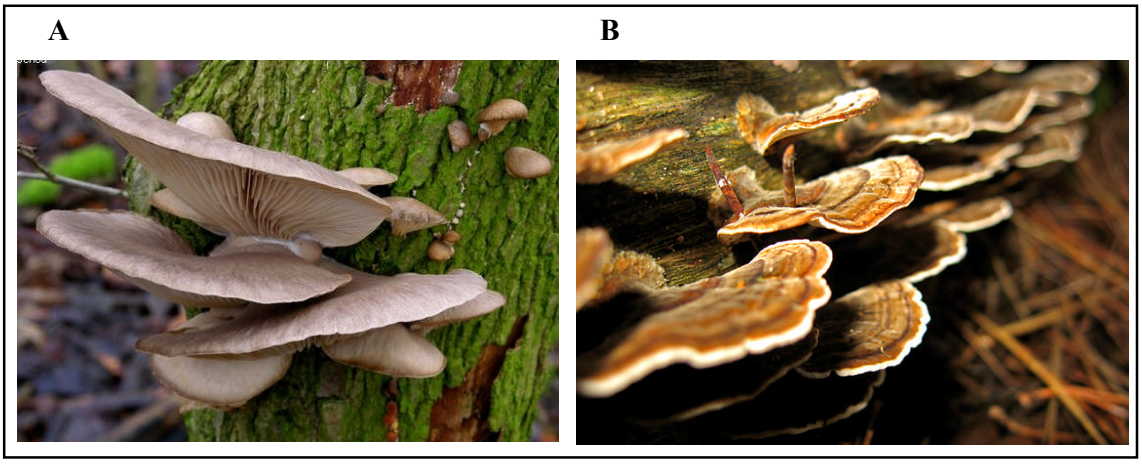

Figure 2.2: Pictures of *Pleurotus ostreatus* (A), and of *Trametes versicolor* (B).

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2.3 Set up of a Solid State Fermentation process

2.3.1 Introduction

This part of the experimental work has dealt with setting up the technique of Solid State Fermentation (SSF), and selecting the parameters that guarantee the success of the developed process, such as substrate particle size, use of a support, substrate water activity, humidity. As previously reported (chapter 1), SSF relies on the ability of many microorganisms to grow in the near/complete absence of free water. Indeed, different classes of microorganisms need different levels of free water in order to grow, thus choosing appropriate process parameters one can select the desired microbial population (i.e.: low levels of free water allow fungal growth, while high levels promote bacterial propagation). In particular, the development of a solid state fermentation process based on fungi seems interesting since SSF conditions are similar to those found in natural fungal environment.

Different process parameters such as particle diameters, air and water availability, influencing gaseous and mass exchanges, play a crucial role in the success of such experiments. Thus, defining appropriate culture conditions seems crucial for the attainment of interesting results. Among the several factors that are important for microbial growth and enzyme production using a particular substrate, particle size and moisture level/water activity are the most critical. Generally, smaller substrate particles provide larger surface area for microbial attack and, thus, are a desirable factor. However, too small substrate particles may result in substrate agglutination, which may interfere with aeration and then with microbial respiration, therefore resulting in poor growth. In contrast, larger particles provide better gaseous exchanges (due to increased inter-particle space), ameliorating microbial respiration, whereas limiting surface for microbial attack. Thus, it is needed to find a compromise about particle size that can fit the requirements of the selected microorganism.

In the present study, a model substrate was selected to be representative of typical greengrocers' wastes and its composition was determined. Being cellulose its prevalent component, the cellulolytic fungus *Thrichoderma harzianum* was chosen, as model conversion microorganism.

2.3.2 Results

The results are reported in the manuscript "Development of fungal Solid State fermentation processes on vegetale wastes" published in the Proceedings book, ITALIC 5, Science & technology of biomasses, Advances and challenges (2009): 238-241

DEVELOPMENT OF FUNGAL SOLID STATE FERMENTATION PROCESSES ON VEGETABLE WASTES*

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Dedicated to the memory of our missed friend and colleague Christopher Augur

Abstract

The possibility to get useful and high-added value products from lignocellulosic wastes makes it possible to transform the huge amounts of residues into an attractive resource (1). In addition to sugars for bioethanol production, bio-colorants, bio-pesticides, enzymes, antioxidants and aromatic compounds can be recovered from vegetable wastes either by extraction or by microbial transformation based on solid-state fermentation of micro-organisms, mainly fungi (2). As a crucial aspect of this study, the ability of filamentous fungi to colonize ligno-cellulosic wastes by solid-state fermentation is exploited to achieve valorisation of vegetable wastes, transforming their macromolecular components in high-added value products. In this study, a model of vegetable wastes has been formulated, taking into account average composition of real greengrocer's wastes. A fungal solid-state fermentation process on the waste model was developed with the cellulolytic fungus *Trichoderma harzianum*, selected because of the prevalent content of cellulose of the waste. Different wastes, such as sugar cane bagasse and olive tree pruning residues, were added to the waste model and tested as supports to improve fungal colonization. The effects of particle dimension, ratio waste model/support, humidity and inoculum conditions were also investigated. Experiments were performed both in flasks and in column reactor (3), and optimal conditions for *T. harzianum* colonization were defined.

Introduction

Huge amounts of wastes having as main component ligno-cellulose and originating from municipal, agricultural, forestry and food farming industry are discharged in Europe, their cost-effective and eco-friendly disposal representing a major concern. The significant amounts of nutrients, mainly carbohydrates, present in these materials makes it feasible to use them as substrates for microbial growth, through solid state fermentation (SSF). This process represents the growth of microorganisms in the absence or near-absence of free water (4, 5), thus being really close to the natural environment in which many higher filamentous fungi have evolved (6). SSF is the focus of increasing attention for conversion of agro-industrial wastes in high-added value products. As a matter of fact many bio-products have been developed from fruit and vegetable residues using the SSF technique, such as ethanol, methane, organic acids, mushrooms, enzymes and food ingredients (7). Several important aspects should to be considered for development of any bioprocess in SSF, such as selection of suitable micro-organism and substrate, optimisation of process parameters and isolation and purification of the product. Among the several factors that are important for microbial growth through SSF on a specific substrate, particle size and moisture level/water activity are the most critical. Particle diameters, air and water availability, that in turn influence gaseous and mass exchanges play a crucial role in the success of such experiments.
In this study, optimal conditions for SSF of the cellulolytic fungus *Trichoderma harzianum* on a model of vegetable wastes were investigated. The effects of particle dimension, ratio green waste/support, humidity and inoculum conditions wereinvestigated, both in flasks and in a column reactor (3). This bioreactor, previously developed by Raimbault and Alazard (3), consists of glass columns with volumes ranging from 95 mL to 1.6 L, placed within a thermostatated bath. The bioreactor is equipped with a system for humidified air supplying and with control of temperature, air flux and humidity.

Experimental

1. Microorganism

Trichoderma harzianum CCM F-470, obtained from Czechoslovak Collection of Microorganisms, Brno, was maintained on potato-dextrose agar at 4°C and by sub-culturing once every four months with incubation at 30 °C. The spores from freshly grown slants were suspended in sterile water containing 0.01% Tween 80 (10 ml/slant) for inoculum preparation.

2. Ligno-cellulosic materials

The fibre (lignin, cellulose and hemicellulose) content of the vegetable residues was determined according to Goering and Van Soest (8). Different vegetable residues were mixed to set up a green waste model to be used as substrate of SSF experiments.

Sugar cane bagasse, collected in Mexico in 1992, was used either as substrate or as support, olive tree pruning residues, collected in Morocco in 2008, were used as support in SSF experiments. Substrate and support materials were both milled and sieved in order to recover particles whose diameter is comprised between 0,8 and 2 mm.

3. Culture conditions

Around 10^{10} spores of *T. harzianum* were obtained after 6 days (with exposure to the light in the last 2-3 days) of growth at 30°C on solid medium containing 2.9% (w/v) potato-dextrose (Difco™) and 1.5% agar (w/v) (Difco™) plates at pH 5.4. The spores were recovered in 0.01% Tween 80 by stirring the suspension in the presence of glass beads and used as inoculum of SSF experiments.

As raw materials for SSF experiments, mixtures of substrate (sugar cane bagasse or green waste model) and support (wheat bran or sugar cane bagasse/olive tree pruning residues) were moistened up to 50% moisture (Roussos et al, 1991). In some cases, addition of a mineral-salt solution of ammonium sulphate, urea and monobasic or dibasic potassium phosphate was performed, selecting its composition to maximise spores germination. After sterilisation by autoclaving at 110°C (0,5 bar) for 1 hour, the substrate/support mixtures with or without glucose addition were inoculated with a suspension of *T. harzianum* spores, whose volume was chosen to reach the required humidity.

As far as SSF experiments in column reactor are concerned, 95 ml columns were used, and the working volume was delimited by filter paper and cotton, placed at the top and at the back of the column. Different amounts (50 or 30 grams) of inoculated moistened mixture of substrate (sugar cane bagasse or green waste model, respectively) and support were subjected to fermentation experiments, taking into account their different texture.

As far as SSF experiments in 250 ml flasks are concerned, 30 grams of inoculated moistened mixture of substrate and support were used.

At three different times during the replenishment of the columns (or flasks) used in a run of SSF experiments, the humidity of material was determined by means of Moisture Meter MA150 (Sartorius Mechatronics Italy S.r.l.), thus estimating the loss of humidity due to the evaporation during the operations.

The fermentation experiments were carried out at at 30°C. Samples were withdrawn each 24 hours for 4-7 days along fermentation and percentage of humidity, pH and weight loss measured on each sample

Results and discussion

1. Formulation of the green waste model as substrate for *T. harzianum* SSF process

A model of vegetable wastes (named green waste model) was formulated, taking into account average composition of real greengrocer's wastes. Analyses of macromolecular composition of vegetable wastes (Table 1) showed a cellulose content of around 20% (w/w) in five out of the 11 analyzed wastes and of 11-15% (w/w) in other 5 wastes, while low levels of lignin and of hemicellulose were detected in most of the analyzed residues. Therefore, equal amounts of black broccoli, chicory, spinach, tricky lettuce, lettuce, green broccoli and cabbage were combined to produce the green waste model, containing 2,61% of lignin, 18,2% of cellulose and 4,2% of hemicellulose.

Vegetable residues	Lignin	Cellulose	Hemicellulose
Basil	11,8	35,1	8,6
Broccoli	2,04	23,46	6
Parsley	3,36	20,84	0,2
Chicory	1,61	20,69	10,7
Lettuce	1,41	18,89	4,2
Tricky Lettuce	0,5	18	5,9
Green Broccoli	0,61	15,69	8,1
Black Broccoli	0,6	14,2	6,6
Cabbage	1,57	13,63	7,1
Spinach	2,21	11,69	13,3
Swiss chard	1,55	10,85	12,3

Table 1. Cellulose, hemicellulose and lignin contents (% w/w) of vegetable residues

Preliminarily, the ability of *T. harzianum* to grow on the green waste model was verified on Petri dishes containing agar added with the green waste model. Germination of spores and growth of mycelium were observed after 29 hours and 49 hours, respectively.

2. Selection of conditions for *T. harzianum* SSF on green waste model

Following the procedure previously developed by Roussos (1987), optimal conditions for fungal SSF were investigated in column reactor (3) with forced aeration, using the green waste model as substrate and sugar cane bagasse as support. The process was compared with *T. harzianum* SSF on sugar cane bagasse supplemented with wheat bran, as support. The substrate green waste model was combined with sugar cane bagasse at a ratio of 85:15 (expressed as percentage of the total dry matter employed), that was selected instead of the ratio of 80:20, used for sugar cane bagasse and wheat bran. The addition of sugar cane bagasse was required due to the lower lignin content of green waste model in order to improve substrate texture for fungal colonization, increasing its rigidity. A humidity of 65% was selected as optimal for SSF on the green waste model instead of 74% (9), due to its lower lignin content responsible for a lower water activity with respect to sugar cane bagasse. Differently for the substrate sugar cane bagasse, it was verified that no addition of any mineral salt solution to the green waste model was required, its pH value (pH 5) after sterilization being close to the pH value optimal for fungal growth. In comparison with the SSF on sugar cane bagasse, an increase of inoculum size (from $2x10^7$ to $1x10^8$ spores per gram of dry material) was required to guarantee a good colonization on the green waste model.

3. SSF process on green waste model in column reactor

During fermentation on the green waste model a constant value of around 65% of humidity was maintained, thanks to the humidified air flux in the column. Similarly, the pH (keeping a constant value of around 4.9 until the 7^{th} day) does not significantly change and just a slight weight loss was measured. Germination of spores started slightly later (at 29 hours) than on sugar cane bagasse (at 26 hours), and mycelium formation was observed at 48 hours. Afterwards, substrate colonization took place with an unhomogeneous distribution, thus requiring a more efficient mixing.

4. SSF process on green waste model in flasks

Fermentation experiments were also performed in flasks at the same conditions selected for column reactor, thus verifying the ability of *T. harzianum* to grow with no significant change in humidity, even in the absence of a flux of humidified air. This behaviour is worth noting since humidity and related water activity are important parameters for gaseous exchanges, microbial growth and waste colonization. The fungus was shown able to colonize the green waste model, mycelium formation being observed at 41 hours and sporulation starting after seven days. Neither weight loss nor pH change (maintaining a costant valure of around 5) were noticed during the fermentation.

Besides sugar cane bagasse, olive tree pruning residues were tested as support of fermentation in flasks. Since olive trees are widely spread in Mediterranean regions, their pruning residues are easier to be found than the wastes from processing of sugar cane, mainly cultivated in regions with equatorial climate.

Two main differences were adopted in culture conditions when olive tree pruning residues instead of sugar cane bagasse were used as support. Addition of a mineral salt solution (50mM dibasic potassium phosphate, 28mM urea, 250mM ammonium sulphate) at pH 7 was needed, since acidification of medium was observed in the presence of olive tree pruning residues. Moreover, 0,1% glucose addition allowed a faster germination of spores, starting at 22-26 hours of fermentation. A slight weight loss was observed, whereas no significant change either of pH or of humidity was measured during fermentation.

Conclusions

In this study, the ability of *T. harzianum* to colonize a model of vegetable wastes (green waste model) was verified and a solid-state fermentation process by the fungus on the waste model was developed both in flasks and in column reactor (3). The green waste model was formulated, taking into account average composition of real greengrocer's wastes, and it consists of vegetables with low levels of lignin. Therefore, the strictly cellulolytic fungus *T. harzianum* was chosen for waste conversion. Waste materials were added as support to help fungal colonization of the substrate material, whose structure is modified by fungal growth. Conditions of fermentation using both sugar cane bagasse and olive tree pruning residues as support were defined, the latter wastes being easier to be found in Mediterranean regions. Moreover, it was crucial to select the composition of a salt solution to bring the substrate to the required pH for fungal growth, taking into account that the process of cooking of vegetables, during autoclaving, can provide a pH change. Finally, appropriate inoculum size and mixing conditions were shown essential requirements to achieve a diffuse colonization and subsequent transformation of model waste.

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2.4 Definition of process conditions allowing the obtaining of high added value products

2.4.1 Introduction

As previously pointed out, lignocelluloses are abundantly produced worldwide, and have been shown to be attractive substrates for the production of industrially relevant products. At this purpose, two different strategies can be pursued, such as the direct extraction of valuable molecules from the raw materials, and the use of wastes as substrates for microbial solid state fermentation (SSF) processes. The main products of lignocellulose transformation by fungal fermentation can be chemicals (i.e.: flavours, colorants, antioxidants), sugars and enzymes (i.e.: oxidases, cellulases, hemicellulases), produced along fungal microbial growth.

In order to obtain the above reported products, the careful selection both of substrates (section 2.1) and microorganisms (section 2.2) are key issues. In this study, tomato and apple processing residues were chosen as substrates. It is worth noticing that they contain industrially relevant molecules, such as polyphenols, pigments and polysaccharides, the latter useful for microbial wastes colonization. The two basidyomicetous fungi *P. ostreatus* and *T. versicolor* were selected as process microorganisms. Moreover, the successful production of the three classes of products will rely on the identification of the appropriate process parameters such as humidity, particles size, inoculum size, etc.. Concerning enzymes production, an advantage of lignocellulsic wastes is that they contain molecules that can induce the production of different classes of industrially relevant enzymes. The possibility of achieving high titers of industrially relevant enzymes is of clue importance, since large amounts of enzymes are required for their applications. As a matter of fact, enzymes production is a growing field of interest in white biotechnology. Annual world sales figures are close to a billion dollars with increasing number of patents and research articles related to this field (Layman, 1990). So far, most enzyme manufacturers produce enzymes using submerged fermentation (SmF) techniques with enzyme titers in the range of grams per liter (Harvey *et al*., 1993). Such levels are a prerequisite if specific compounds are to be considered as commodities, because product recovery costs are inversely proportional to their concentration in the fermentation broth (Kroner *et al*., 1984). There is, however, a significant interest in using solid state fermentation (SSF) techniques to produce a wide variety of enzymes as indicated by the growing number of research papers in the literature (Pandey *et al*., 1999).

As before mentioned, the high requirements of enzymes are due to their large use in industrial processes, in different fields, ranging from food and beverages industry, to leather and paper processings. As well, enzymes are being increasingly employed in bioremediation.

Laccases are phenol-oxidasing enzymes, being mainly exploited for white biotechnology applications such as removal of phenolic pollutants, treatment of dyes containing wastewaters (Rodriguez Couto *et. al*., 2006a) and in industrial processes (biobleaching, biopulping, etc.) (Rodriguez Couto *et al*., 2006b, Rodriguez Couto, 2008). Other oxidative enzymes, MnP and LiP have been mainly used in the treatment of olive mill waste waters, for the removal of synthetic dyes, and other xenobiotics.

Among hydrolytic enzymes, proteases show important roles in several physiological processes based on their catalytic activity of cleavage of peptide bonds. They constitute a large family (EC 3.4) such as endopeptidases or proteinases (EC 3.4 21-

99) and exopeptidases (EC 3.4.11-19) depending on the point at which they break the peptide chain. They represent one of the three largest groups of industrial enzymes, accounting for about 60% of the total worldwide sale of enzymes, having huge commercial applications. They are envisaged to have extensive applications in leather treatment, in pharmaceutical industry and in several bioremediation processes.

Finally, glycosyl hydrolases find several applications in different areas. Pectinolytic enzymes are required for extraction and clarification of fruit juices and wines, extraction of oils, flavours and pigments from plant materials, preparation of cellulose fibers for linen, jute and hemp manufacture, in coffee and tea fermentations (Taragano *et al*., 1997) and in the production of oligogalacturonides as functional food components (Hang *et al*., 2000).

The main industrial application of cellulases is the hydrolysis of lignocellulosic biomasses into sugars, to be further converted, by microbial fermentation, in several marketable bioproducts (biopolymers, fuel ethanol production, etc.). They have, also, proved commercially useful catalysts in textile, detergent and pulp and paper industries (Gupta, 2002).

The past two decades have seen a growing interest in microbial enzyme systems that degrade plant xylan, being xylan the second most abundant biopolymer in lignocellulose after cellulose. Xylanases addition to cellulolytic mixtures can improve the overall yield of sugars for industrial fermentations. Xylanases have potential applications in the pulp and paper, food, beverages and feed industries (Subramaniyan *et al*., 2002, Wong *et al*., 1992, Buchert *et al*., 1993, Senior *et al*., 1991). Xylanases free of cellulases can be exploited in the pulp and paper industry, allowing to reduce the use of chemicals. Xylooligosaccharides, produced by the action of these enzymes, can be used as food additives (Koga *et al.*, 1993). Other applications of xylanolytic enzymes include improvement of baking process and modification of baked products. Xylanases are also important components of enzyme systems used for liquifaction of vegetables and fruit, and for clarification of juices (Biely, 1985, Wong *et al*., 1993). Pretreatment of forage crops with xylan-degrading enzymes improves the nutritional quality and digestibility of ruminant fodder and facilitates composting (Wong *et al*., 1993, Gilbert *et al*., 1993).

α-L-arabinofuranosidase can be used to enhance wine flavour by release of free terpenols (Gunata *et al*., 1988), since most terpenols are linked to disaccharides moieties in which the non-reducing terminal sugar is often L-arabinofuranose.

Among the above mentioned classes of enzymes, we took into account laccases, peroxidases, xylanases, cellulases, and proteases, highlighting the conditions that favour their production in SSF experiments of *P. ostreatus* and *T. versicolor* on tomato and apple pomaces.

2.4.2 Results

The results of this part of the project have been divided into the following sections:

2.4.2.1 Solid state fermentation for tomato pomace valorization,

- 'High added value products from ligno-cellulosic wastes though fungal Solid State fermentation processes' in Proceedings book, ITALIC 5, Science & technology of biomasses, Advances and challenges (2009): 213-216
- 'Enzyme production by solid substrate fermentation of *Pleurotus ostreatus* and *Trametes versicolor* on tomato pomace' submitted to Biochemical Engineering Journal.

2.4.2.2 Solid state fermentation for apple pomace valorization.

HIGH ADDED VALUE PRODUCTS FROM LIGNO-CELLULOSIC WASTES THROUGH FUNGAL SOLID STATE FERMENTATION PROCESSES

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Abstract
A process of fungal solid state fermentation (SSF) on ligno-cellulosic wastes was developed in order to transform them in high added value products (1). Agro-industrial residues were selected as raw materials for fungal solid state fermentation, and a process for valorisation of tomato processing residues by SSF with the fungus *Trametes versicolor* was developed. Around 6 million tons of tomato were processed at industrial scale in Italy in 2005, and 500 thousand tons of these were transformed in the Campania Region (South-Italy) (2). Processing of tomato is characterized by a product/waste ratio of around 40 (3), thus giving raise to the production of about 12.000 tons of wastes in Campania in the year 2005. Tomato processing residues for SSF experiments reported in this study were collected in a local farm in Campania. Operative parameters that guarantee a good colonization of tomato processing residues by *T. versicolor* SSF were defined and conditions in which high levels of laccase and xylanase activity production are achieved were identified.

Introduction
Solid-state fermentation (SSF) represents the growth of microorganisms in the absence or near-absence of free water (4,5). As pointed out by Pandey et al. (6), it's amenable to distinguish solid state fermentation and solid substrate fermentation. Both processes occur in the absence or near-absence of free water, but the latter should be used to define only those processes in which the substrate itself acts as carbon/energy source. Solid state fermentation should define any fermentation process employing a natural substrate as above, or an inert substrate used as solid support. Since the last decade, many studies about the application of SSF were focused on adding an extra value to agro-industrial residues and several processes have been developed aimed at enzyme production and metabolite synthesis (7). The selection of a substrate for enzyme production in a SSF process depends upon several factors, mainly related to cost and availability of the substrate, and thus may involve screening of several agro-industrial residues. Agro-industrial residues are generally considered the best substrates for SSF processes, and a number of such substrates have been employed for the cultivation of micro-organisms to produce enzymes (8). Tomato processing residues represent one of the most abundant wastes of agro-industrial sector. In 2005, 10 million tons of tomato were processed in Europe (http://www.bioactive-net.com), 6 million tons of these were transformed in Italy, where the Campania Region (South-Italy) contributed with 500 thousand tons of processed tomato (2). A product/waste ratio of around 40 (3) for tomato processing gives a total amount of solid tomato residues (peels and seeds, called pomace) of about 12.000 tons in this region. However, very few manuscripts were so far reported on conversion of tomato pomace, animal feeding being the main way currently adopted for its disposal.
In this study, operative parameters that guarantee a good colonization of tomato processing residues by *T. versicolor* SSF were defined and conditions in which high levels of laccase and xylanase activity production are achieved were identified.

Experimental

2.1. Lignocellulosic substrates

Commercially available wheat employed for fungal pre-culture was weighted, washed, and moisturized with bi-distilled water (1:1, weight/volume) before autoclaving for 1 hour at 110 °C. After the addition of 1 % (w/w) $CaCO_3$ and of bi-distilled water (1:1, weight/volume), a new sterilization cycle was run.

Tomato residues were collected in a local tomato processing farm in Campania (Italy). The residues (peels and seeds) were dried at 65 °C, reduced in small pieces and sieved to have 0.8–2.0 mm dimension particles. Sorghum stalks, after milling and sieving, were added to tomato residues up to 15 % of the total dry matter. Before sterilizing in autoclave for 1 hour at 110 °C, a moisture content of 65 % (v/w) was achieved by water addition and a final pH of 6.0 was obtained by adding 1% (w/w) $CaCO_3$.

2.2. Fungal strains and culture conditions

The strain NBRC4937 of *Trametes versicolor*, purchased from the fungal collection Nite Biological Resource Center (Department of Biotechnology, National Institute of Technology and Evaluation Kisarazu, Chiba, Japan), was used in this study. The fungus was maintained through periodic transfer at 4 °C on PDY agar [(24 g/l potato dextrose (Difco, Detroit, Michigan, USA), 5 g/l yeast extract (Difco), agar (1.5 % w/v)] plates. Pre-cultures were prepared by pre-inoculating 100 g of pre-treated wheat in 1 L Erlenmeyer flasks with 10 agar (9 mm diameter) plugs of *T. versicolor* mycelium, from the edge of a 6-days-old agar culture. After fungal growth for six days in a temperature-controlled incubator at 28°C, the wheat mechanically stirred was used to inoculate 500 g of wet tomato waste prepared as above described. 50 g of the material, made homogeneous by stirring, were transferred in 250 ml flasks, then incubated at 28°C, statically. The fermentation was followed for 18 days, and at suitable time intervals, three flasks were sampled. The content of each flask was used to prepare samples for the analytical determinations. Lyophilized and milled samples were suspended (1:10 weight/weight) in bi-distilled water for reducing sugars determination and in 50 mM $NaPO_4$ pH 6.8 added with 1mM phenylmethylsulfonylfluoride (PMSF), for enzymatic assays. After vigorous mixing by vortex, samples were centrifuged for 30 minutes at 7500 rpm at 4°C (Beckman Coulter, Inc.), and supernatants were subjected to analytical determinations, as below described. The experiments were performed by using three replicates.

2.3. Analytical procedures

2.3.1 Humidity and weight loss evaluation

The percentage of humidity was determined gravimetrically. In a pre-weighed glass plate (W1) 1-2 grams of sampled material (W2) were allowed to dry in a ventilated oven at 65°C for about 24 hours. After cooling in a dessiccator, plates were weighed to get the dry weight (W3). Percentage of humidity was evaluated as {[(W2-W1)-(W3-W1)]/ (W2-W1)} *100

To determine weight loss at each sampling time, the change of total dry matter weight was measured with respect to the beginning of fermentation. Data were reported as percentage of the initial dry matter content.

2.3.2 Analysis of soluble reducing sugar content

Reducing sugars were measured by the 3,5-dinitrosalicylic acid (DNS) method using (100-500 µg) D-glucose as a standard, according to Miller (9). Sugar content was assayed on 0.5 ml of the appropriately diluted sample. A DNS solution containing 10 g/L 3,5-dinitrosalicylic acid (Sigma Aldrich), 10 g/L sodium hydroxide, 0.5 g/L anhydrous sodium sulfite and 2g/L phenol was prepared. A 1:1 (volume/volume) mixture of diluted sample and DNS solution was incubated at 95°C for 5 minutes, after mixing by vortex. Assay mixture was let cool and, after addition of 3 ml of bi-distilled water, absorbance was measured at 580 nm.

2.3.3 Enzyme assays

a) Laccase activity

Laccase activity was assayed using 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate (10). The assay mixture contained 2 mM ABTS and 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}). Enzyme activity was expressed in international units (IU).

b) Filter paper activity Filter paper activity was assayed by a modified version of protocol reported by Ghose (11). A strip of 50 mg (1x 6 cm) of Whatman #1 filter paper was used as a substrate, by

incubating it for 1hour at 50°C, in a mixture of 1 ml of 50 mM sodium citrate pH 4.8 and 0.5 ml of the appropriate sample dilution (in 50 mM sodium citrate at pH 4.8). 0.5 ml of this reaction mixture were used to determine the released reducing sugars, by DNS method. A mixture of the analyzed sample and DNS solution (1:1, volume/volume) was incubated at 95°C for 5 minutes. Then, the assay mixture was let cool and, after addition of 3 ml of bi-distilled water, absorbance was measured at 580 nm. Enzyme activity was inferred using D-glucose as a standard.

c) Xylanase activity

Xylanase activity assay was performed according to Bailey et al. (12). The reaction mixture consisting of 1.8 ml of a 1.0% (w/v) suspension of birch-wood xylan in 50 mM Na Citrate at pH 5.3 and 0.2 ml of enzyme dilution (in 50 mM sodium citrate at pH 5.3) was incubated at 50°C for 5 min. Released reducing sugars were determined by DNS method, by adding 3 ml of DNS solution and then incubating the mixture at 95°C for 5 minutes. Absorbance was measured at 540 nm. One unit of enzyme is defined as the amount of enzyme catalyzing the release of 1 µmol of xylose equivalent per min.

2.3.4 Evaluation of total protein content

Protein concentration was determined using the BioRad Protein Assay (BioRad), with bovine serum albumin as standard.

Results and discussion

T. versicolor was investigated for its ability to colonize tomato processing wastes (peels and seeds), using them as substrates for solid state fermentation. Appropriate conditions for fungal growth and waste colonization were investigated. Optimal values of particles dimension, substrate/support ratio, humidity, pH and appropriate inoculum conditions were selected.

Waste colonization by *T. versicolor* was observed beginning from the 2nd day of fermentation. No significant change of humidity was measured during fermentation. Time courses of weight loss and sugar content were analysed during SSF. An increasing weight loss was measured until the 10th day. Beginning from this day, a constant value of weight loss related to a stationary phase of fungal growth was detected. A fast decrease of sugar content was measured until the 4th day, followed by a stationary level of sugar content.

T. versicolor was assessed for its ability to produce laccases and glycosyl hydrolases (xylanases and cellulases) during SSF on tomato wastes.

A maximum laccase activity production of 35 U/ g dry wt was achieved. This level of laccase activity production is similar or higher than those so far reported in other fungal SSF experiments. Elisashvili et al. (13) reported a laccase activity production of 14 U/ g of substrate, when SSF of the fungi *Lentinula edodes, Pleurotus drynus and Pleurotus ostreatus* were carried out on tree leaves or on wheat straw. Rodriguez-Couto et al. (14) obtained 40 U/g of substrate at the 13th day of *Trametes hirsuta* SSF on grape seeds moistened with a synthetic medium in a tray 1.8 L bioreactor. Lower levels (0.040 IU/g dry weight) of laccase activity were achieved in SSF at the 8th day of *P. ostreatus* SSF on sugar cane bagasse in the presence of ammonium sulphate (15).

Among the assayed glycosyl hydrolases, cellulase and xylanase, the latter enzyme activity was produced at higher levels, reaching a maximum value of around 50 U/g dry matter. Highly variable levels of xylanase activity production were previously measured when different fungi were analyzed in SSF experiments (13), with a maximum production of around 70 U/ g, 536 U/ g and 546 U/ g of substrate for *L. edodes* (on wheat straw), *P. drynus* and *P. ostreatus* (on tree leaves), respectively. Around 5 U/g dry weight of xylanase activity were obtained in SSF at the 8th day of *P. ostreatus* SSF on sugar cane bagasse in the presence of ammonium sulphate (15).

Conclusions

A process of fungal solid state fermentation on tomato processing wastes (peels and seeds) was for the first time developed, exploiting ability of *T. versicolor* to colonize the waste. Appropriate conditions for fungal growth and waste colonization were selected. Results highlight the potential of the developed process for valorisation of tomato wastes as a source of the industrially relevant enzymes xylanase and laccase. The set up tomato waste conversion process can represent a cost effective system for enzyme production requiring low technical and economical inputs. As a matter

of fact, in our study significant enzyme production levels were achieved from tomato waste without optimization of culture conditions neither by nutrient addition nor by O_2 enrichment.

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Abstract: A process of solid state fermentation (SSF) on tomato pomace was developed with the white-rot fungi Pleurotus ostreatus and Trametes versicolor. Operative parameters (humidity, water activity, size of substrate particles) guaranteeing a good colonization of tomato pomace by both fungi were defined and conditions for production of the industrially relevant enzymes laccase, xylanase and protease were identified. Significant laccase activity levels (up to 36 U g-1 dry matter) were achieved without any optimization of culture conditions, neither by nutrient addition nor by O2 enrichment. Furthermore, protease activity levels up to 34,000 U g-1 dry matter were achieved, being higher than those reported for the fungi typically considered as the best protease producers such as Aspergillus strains. Moreover, as one of the most significant results of this study, analysis of P. ostreatus tomato SSF samples by zymogram revealed two bands with laccase activity which had not been detected so far.

Enzyme production by solid substrate fermentation of *Pleurotus ostreatus* and *Trametes versicolor* on tomato pomace

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Abbreviated title: Fungal fermentation on tomato pomace

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Abstract
A process of solid state fermentation (SSF) on tomato pomace was developed with the white-rot fungi *Pleurotus ostreatus* and *Trametes versicolor*. Operative parameters (humidity, water activity, size of substrate particles) guaranteeing a good colonization of tomato pomace by both fungi were defined and conditions for production of the industrially relevant enzymes laccase, xylanase and protease were identified. Significant laccase activity levels (up to 36 U g^{-1} dry matter) were achieved without any optimization of culture conditions, neither by nutrient addition nor by O_2 enrichment. Furthermore, protease activity levels up to 34,000 U g^{-1} dry matter were achieved, being higher than those reported for the fungi typically considered as the best protease producers such as *Aspergillus* strains. Moreover, as one of the most significant results of this study, analysis of *P. ostreatus* tomato SSF samples by zymogram revealed two bands with laccase activity which had not been detected so far.

Keywords: fungi; solid state fermentation**;** laccase; xylanase; protease.

1. Introduction

Among biotechnology products, enzymes encompass one of the biggest market share with considerable selling prices entailing high costs in the following industrial processes. Worldwide enzyme production is mainly based on microbial submerged fermentation (SmF) processes, with high production costs due to the exploitation of genetically modified microorganisms, expensive culture conditions and downstream processing for product recovery. Microbial solid state fermentation (SSF) holds tremendous potential for enzyme production, being a better system than SmF from both economical and environmental points of view [1, 2]. SSF involves the growth of microorganisms in the absence or near-absence of free water [3, 4]. Basidiomycetous fungi represent the most appropriate microorganisms for producing enzymes through SSF, because of similarity between their natural environment and the conditions in which SSF processes are carried out [1, 2]. Agro-industrial residues are generally considered the best substrates for SSF processes and many studies have being

focused on application of SSF to adding extra value to these wastes through enzyme production and metabolite synthesis [5-7]. Different organic wastes such as bagasse, ballico seed, banana waste, canola roots, corn, cotton, grape, sawdust, wheat bran, wheat straw and wood have shown to be good substrates for enzyme production by fungal SSF, due to the presence of significant concentrations of soluble carbohydrates and inducers of enzyme synthesis [8, 9]. Till now, several industrially relevant enzymes have been obtained through fungal SSF on agricultural and food farming residues, mainly belonging to the class of ligninolytic enzymes, such as laccases, Manganese Peroxidases (MnP) and Lignin Peroxidases (LiP) [10], and of carbohydrate hydrolysing enzymes, such as amylases, xylanases, cellulases and pectinases [11]. Obtaining good yields of other enzymatic activities such as proteases, chitinases and tannases has also been achieved by fungal SSF [12].
Tomato processing residues represent one of the most abundant wastes of agro-industrial sector . In 2005, 10 million tons of tomato were processed in Europe (www.bioactive-net.com, 20/09/2009) and 6 million tons of these were transformed in Italy, where the Campania Region (South-Italy) contributed with 500 thousand tons of processed tomato (www.anicav.it, 20/09/2009). A ratio of transformed product to waste of around 50 [13] gives a total amount of solid tomato residues (peels and seeds, called pomace) of about 10 thousand tons in this region. Animal feeding represents the main way currently adopted for disposal of tomato pomace, whilst very few studies have been reported so far on conversion of this waste, such as its fungal transformation to improve digestibility [14].
In this study, operative parameters that guarantee a good colonization of tomato pomace by SSF of the basidiomycetous fungi *Pleurotus ostreatus* and *Trametes versicolor* were defined and conditions for laccase, xylanase and protease activity production were identified. SSF of *P. ostreatus* on tomato pomace led to the production of laccase isoforms not detected in any of the conditions which have been analysed so far.

2. Materials and methods

2.1. Lignocellulosic substrates

Commercially available wheat employed for fungal pre-culture was weighted, washed, and moisturized with bi-distilled water (1:1, weight/volume) before autoclaving for 1 hour at 110 °C. After the addition of 1 % (w/w) $CaCO_3$ and of bi-distilled water (1:1, weight/volume), a new sterilization cycle was run.
Tomato residues were collected in a local tomato processing farm in Campania (Italy). The residues (peels and seeds) were dried at 65 °C, reduced in small pieces and sieved to have 0.8–2.0 mm dimension particles. Sorghum stalks, after milling and sieving, were added to tomato residues up to 15 % of the total dry matter. Before sterilizing in autoclave for 1 hour at 110 °C, a moisture content of 65 % (w/w) was achieved by water addition and 1% (w/w) $CaCO_3$ was added in order to get a final pH of 6.0 after sterilization.

2.2. Fungal strains

Strains of the white rot fungi *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC MYA-2306) from ATCC, the Global Bioresource Centre and *T. versicolor* (NBRC4937) from the fungal collection Nite Biological Resource Center (Department of Biotechnology National Institute of Technology and Evaluation, Japan) were used in this study.
The fungi were maintained through periodic transfer at 4 °C on agar (1.5% w/v) plates containing PDY medium [24 g l^{-1} potato dextrose (Difco, Detroit, Michigan, USA) and 5 g l^{-1} yeast extract (Difco)].

2.3 Solid state fermentation

Pre-cultures were prepared by pre-inoculating 100 g of pre-treated wheat in 1 L Erlenmeyer flasks with 5 agar plugs of *P. ostreatus* (11 mm diameter) or 10 agar plugs of *T. versicolor* (9

mm diameter) mycelia, from the edge of a 5-days-old agar culture. The different pre-cultures conditions were chosen to achieve a comparable growth for both the fungal strains.
After fungal growth in a temperature-controlled incubator at 28°C for six days, the wheat, after mechanical stirring, was used to inoculate 500 g of wet tomato waste prepared as above described. 50 g of the material, made homogeneous by stirring, were transferred in 250 ml flasks, then incubated at 28°C, statically. The fermentations of *P. ostreatus* and *T. versicolor* were followed for 18 and 22 days, respectively, due to the different grow rates of the analysed microorganisms. At suitable time intervals, three flasks were sampled and the content of each flask was used to prepare samples for the analytical determinations. Freeze dried and milled samples were suspended (1:10 weight/weight) in bi-distilled water for reducing sugars determination and in 50 mM Sodium Phosphate pH 6.8 and added with 1mM phenylmethylsulfonylfluoride (PMSF) for enzymatic assays. When sampes were used for protease activity assay, PMSF was not added to the proteic extract. After vigorous mixing by vortex, samples were centrifuged for 30 minutes at 7500 rpm at 4°C (Beckman Coulter, Inc.), and supernatants were subjected to analytical determinations, as below described.
The results of the analytical determinations reported in the figures correspond to mean values of the three-replicates with a standard deviation lower than 15%.

2.4. Analytical procedures

2.4.1 Humidity and weight loss evaluation

The percentage of humidity was determined gravimetrically. In a pre-weighed glass plate (W1) 1-2 grams of sampled material (W2) were allowed to dry in a ventilated oven at 65°C for about 24 hours. After cooling in a dessiccator, plates were weighed to get the dry weight (W3). Percentage of humidity was evaluated as {[(W2-W1)-(W3-W1)]/ (W2-W1)} *100.
To determine weight loss at each sampling time, the change of total dry matter weight was measured with respect to the beginning of fermentation. Data were reported as percentage of the initial dry matter content.

2.4.2 Analysis of soluble reducing sugar content

Reducing sugars were measured by the 3,5-dinitrosalicylic acid (DNS) method using (100-500 µg) D-glucose as a standard, according to Miller [15]. Sugar content was assayed on 0.5 ml of the appropriately diluted sample. A DNS solution containing 10 g l^{-1} 3,5-dinitrosalicylic acid (Sigma Aldrich), 10 g l^{-1} sodium hydroxide, 0.5 g l^{-1} anhydrous sodium sulfite and 2 g l^{-1} phenol was prepared. A 1:1 (volume/volume) mixture of diluted sample and DNS solution was incubated at 95°C for 5 minutes, after mixing by vortex. Mixtures were let to cool to room temperature and, after addition of 3 ml of bi-distilled water, absorbance was measured at 580 nm.

2.4.3 Enzyme assays

a) Laccase activity

Laccase activity was assayed using 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate [16]. The assay mixture contained 2 mM ABTS and 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}).

b) Manganese peroxidase

Manganese peroxidase (MnP) activity was determined using manganese sulfate as substrate [17]. The reaction mixture contained 0.5 mM manganese sulfate and 0.1 mM Hydrogen peroxide in 50 mM sodium malonate buffer, pH 4.5. Oxidation of Mn^{2+} to Mn^{3+} was followed by absorbance increase at 270 nm (ε = 11,590 M^{-1} cm^{-1}) due to the formation of malonate–Mn^{3+} complex.

c) Lignin peroxidase

Lignin peroxidase (LiP) activity was determined using veratryl alcohol as substrate [18]. The reaction mixture contained 2 mM veratryl alcohol and 0.5 mM hydrogen peroxide in 50 mM

Sodium tartrate buffer, pH 2.5. Oxidation of veratryl alcohol was followed by measuring the absorbance increase at 310 nm (ε = 9,300 M^{-1} cm^{-1}).

All the enzyme activities were expressed in International Units (IU), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes one µmole of substrate in 1 min.

d) Filter paper activity

Filter paper activity was assayed by a modified version of protocol reported by Ghose [19]. A strip of 50 mg (1x 6 cm) of Whatman #1 filter paper was used as a substrate, by incubating it for 1hour at 50°C, in a mixture of 1 ml of 50 mM sodium citrate pH 4.8 and 0.5 ml of the appropriate sample dilution (in 50 mM sodium citrate at pH 4.8). 0.5 ml of this reaction mixture were used to determine the released reducing sugars, by DNS method. A mixture of the analyzed sample and DNS solution (1:1, volume/volume) was incubated at 95°C for 5 minutes. Then, the assay mixture was let cool and, after addition of 4 ml of bi-distilled water, absorbance was measured at 580 nm. Enzyme activity was inferred using D-glucose as a standard.

e) Xylanase activity

Xylanase activity assay was performed according to Bailey et al. [20]. The reaction mixture consisting of 1.8 ml of a 1.0% (w/v) suspension of birch-wood xylan in 50 mM Na citrate at pH 5.3 and 0.2 ml of enzyme dilution (in 50 mM sodium citrate at pH 5.3) was incubated at 50°C for 5 min. Released reducing sugars were determined by DNS method, by adding 3 ml of DNS solution and then incubating the mixture at 95°C for 5 minutes. Absorbance was measured at 540 nm. One unit of enzyme is defined as the amount of enzyme catalyzing the release of 1 µmol of xylose equivalent per min.

f) Protease activity

The substrate used for protease activity determination is azoalbumin (Sigma-Aldrich). The substrate stock solution (15 mg ml^{-1}) was prepared in 3-(N-Morpholino)-propanesulfonic acid (MOPS) buffer 50 mM pH 7. 250 µl of appropriately diluted sample were incubated, in 24 wells plates, with 400 µl of substrate solution for 30 min at 37°C. Reaction was stopped by adding trichloroacetic acid 20% (650 µl). The mixture was centrifuged (600g x 15 min), and 325 µl of Sodium hydroxide 10M were added to 650 µl of digested substrate. Absorbances were registered at 440 nm, against a blank constituted by substrate and MOPS, in the same proportion used for the reaction. One unit of enzyme is defined as the amount of enzyme catalysing the release of the amount of azodye producing 0.001 unit change in the absorbance read at λ=440 nm.

2.5 Evaluation of total protein content

Protein concentration was determined using the BioRad Protein Assay (BioRad), with bovine serum albumin as a standard.

2.6 Non-denaturing PAGE

Polyacrylamide gel electrophoresis (PAGE) was performed at an alkaline pH under nondenaturing conditions. The separating and stacking gels contained 9 and 4% acrylamide, respectively. The buffer solution used for the separating gel contained 50 mM Tris hydrochloride (pH 9.5), and the buffer solution used for the stacking gel contained 18 mM Tris hydrochloride (pH 7.5). The electrode reservoir solution contained 25 mM Tris hydrochloride and 190 mM glycine (pH 8.4). The gels were stained to visualize laccase activity by using ABTS as the substrate.

3. Results

The basidiomycetous fungi *P. ostreatus* and *T. versicolor* were analysed for their ability to colonize tomato pomace, using this waste as a substrate for SSF. Appropriate conditions for

cultivation of fungi on tomato pomace collected in a local farm of the Campania region (South Italy) were investigated and values of particles dimension, substrate/support ratio, humidity and pH and inoculum conditions allowing fungal waste colonization were identified, as reported in the sections 2.1 and 2.3.

A good colonization of tomato waste was achieved with both *P. ostreatus* and *T. versicolor*, fungal growth being observed beginning from the 2nd day of fermentation. No significant change in humidity was measured during fermentation with both fungi (data not shown).

Time courses of weight loss (%) and sugar content (mg g^{-1} of dry matter, mg g^{-1} d.m.) were analysed during SSF (Fig. 1). A continuous weight loss (%) was measured for both *P. ostreatus* (Fig. 1A) and *T. versicolor* (Fig. 1B) SSF, up to around 40% and 20%, respectively. No significant change of sugar content was observed for *P. ostreatus* (Fig. 1A), whereas *T. versicolor* SSF (Fig. 1B) gave rise to a sugar content decrease until the 4th day, followed by stationary levels.

Production of ligninolytic (laccases, LiP and MnP), glycosyl hydrolytic (xylanases and cellulases) and proteolytic enzymes by *P. ostreatus* and *T. versicolor* during SSF on tomato pomace was evaluated.

No peroxidase activity was detected during both *P. ostreatus* and *T. versicolor* fermentations. A maximum laccase activity production level of 15 U g^{-1} of dry matter (U g^{-1} d.m.) was achieved at the 3rd – 4th day of *P. ostreatus* fermentation, as shown in Fig 2A. During *T. versicolor* SSF, a maximum laccase activity value of around 35 U g^{-1} d.m. was produced at the 16th day, keeping constant at least until the 22nd day (Fig. 2B).

Among the assayed glycosyl hydrolases, no detectable levels of cellulase were achieved with both fungi. As far as xylanase activity production is concerned, a maximum activity level of around 9 U g^{-1} d.m. was reached at the 2nd day of *P. ostreatus* SSF (Fig. 3A), whereas a higher level of about 50 U g^{-1} d.m. was achieved at the 13th day of *T. versicolor* SSF (Fig. 3B).

Analyses of protease production by *P. ostreatus* showed achievement of a maximum activity value of around 13,000 U g^{-1} d.m. after only 4 days (Fig. 4A), while a higher protease activity level of 20,000 U g^{-1} d.m. was measured for *T. versicolor* fermentation since its beginning with a subsequent increase up to around 35,000 U g^{-1} d.m. (Fig. 4B).

Production of laccase and protease activities in SSF and liquid culture of *P. ostreatus* in PDY medium [21] were compared (Fig. 5, 6 and 7).

4. Discussion

In this study, a process of fungal solid state fermentation on tomato pomace was for the first time investigated for the production of industrial enzymes, exploiting the natural ability of the fungi *P. ostreatus* and *T. versicolor* to colonize lignocellulosic wastes by SSF. The weight loss measured during fungal fermentations could be ascribed to waste conversion into volatile compounds produced by fungal metabolism. *T. versicolor* was shown to be able to metabolize the sugars present in the waste, giving rise to a fast sugar consumption followed by an increase of xylanase activity level (Fig. 3B). Hence, the fungus seems to react to sugar depletion by producing enzymatic activities involved in polysaccharide hydrolysis. On the other hand, *P. ostreatus* seems not able to metabolize the monosaccharides present in the waste, and the absence of sugars to be directly metabolized could trigger the fungus to produce xylanases in a shorter time than *T. versicolor* (Fig. 3A), thus gaining the required carbon source.

Comparing *T. versicolor* and *P. ostreatus* enzyme production, for all the assayed enzyme activities higher maximum values were achieved with *T. versicolor,* but longer times were needed. Moreover, at the beginning of fermentation higher values of both xylanase and

protease activities were measured for *T. versicolor*, suggesting a higher enzyme production even during pre-culture of this fungus.

For both fungi, the correlation between production times for laccase and xylanase activities suggest their synergistic action in waste transformation.

Lignino-cellulolytic enzyme production by *P. ostreatus* is mainly affected by the strain, substrate composition and culture conditions [22]. In comparison with other *P. ostreatus* strains previously cultivated under SSF conditions, the strain investigated in this study allowed the achievement of higher levels of laccase production after a shorter fermentation time. Kerem et al. [23] have reported a maximal laccase activity production of 0.03U U g^{-1} at the 6^{th} day of *P. ostreatus* SSF on cotton stalks, and similar values (0.040 U g^{-1}) have been achieved by Membrillo et al. [24] at the 8^{th} day of *P. ostreatus* SSF on sugarcane bagasse. Higher laccase activity levels (6 and 4 IU g^{-1}) have been obtained by Stajic et al. [22] and Elisashvili et al. [25] after 10 days of *P. ostreatus* SSF on grapevine sawdust (supplemented with a synthetic medium) and wheat-straw (supplemented with mineral salts and yeast extract), respectively.

Even when compared to SSF processes so far developed with other fungi, the maximum laccase activity levels achieved in this study with *T. versicolor* and *P. ostreatus* were proved significant. Comparing *Lentinus edodes*, *P. dryinus*, *P. ostreatus* and *P. tuberregium* SSFs on wheat straw and tree leaves, Elisashvili et al. [25] have reported a maximum laccase activity value of 14 U g^{-1} by *L. edodes* SSF on both substrates supplemented with mineral salts and yeast extract. By screening different food (apple, orange and potato) wastes as substrates for *T. hirsuta* SSF, Rosales et al. [26] have found a maximum laccase activity value of about 7.5 U g^{-1} within 8 days of fermentation on potato peelings. Grape seeds have shown to be a better substrate for laccase production by *T. hirsuta* SSF, giving 69 U g^{-1} on the 15^{th} day of SSF in the presence of thiamine [27] and 40 U g^{-1} at the 13^{th} day of SSF in the presence of a synthetic medium in a tray 1.8 l bioreactor [28]. Rosales et al. [29] have reported laccase production by *T. hirsuta* SSF on kiwi fruit wastes, with a maximum activity value of around 32.4 U g^{-1} in optimized operative conditions, including initial concentration of ammonium, amount of support employed, need for pre-treating the support and the part of kiwi fruit wastes used (peelings or peelings plus pulp). The achievement of a maximum laccase activity of 4.5 U g^{-1} on banana skin has been reported for *T. pubescens* SSF [30]. Comparing fifteen fungal strains in SSF on three industrial waste materials (oat husks and waste from paper process industry fibre sludge and combined fibre and de-inking sludge -FDS-), Winquist et al. [31] have achieved the maximum laccase activity level (10.65 U g^{-1}) with *Cerrena unicolor* T 71 on the medium containing oat husks or oat husks supplemented with 20% (w/w) of FDS. When a comparative study on *Pycnoporus sanguineus* SSF has been carried out by Vikineswary et al. [32] (on sago hampas, oil palm frond parenchyma tissue -OPFPt- and rubberwood sawdust), sago hampas and OPFPt have shown to be the best substrates, giving a maximum laccase production of 46.5 U g^{-1} at the 11^{th} day of fermentation, through optimization of the inoculum age, density and nitrogen supplementation.

Hence, when compared to the previously reported data, results highlight the great potential of the developed *P. ostreatus* and *T. versicolor* SSF processes for valorisation of tomato wastes as a source of laccases, taking into account that no optimization of culture conditions was performed in this study.

Furthermore, it is worth noting that the laccase activity levels produced by SSF fermentation with the *P. ostreatus* strain used in this study appear significant even when compared with its laccase production in liquid culture. As shown in Fig. 5, laccase activity produced by *P. ostreatus* during SSF and reported as U mg^{-1} of total secreted protein is almost 3 fold higher than that obtained in liquid culture (PDY). Moreover, as shown in Fig. 6, native PAGE analysis of the laccase isoenzymes produced by *P. ostreatus* SSF revealed two more bands

besides the three isoenzymes normally secreted in liquid cultures [16, 21, 33-35]. The existence of these new isoenzymes is consistent with the multiplicity of *P. ostreatus* laccase genes [36] (http://genome.jgi-psf.org/PleosPC15_1, 27/10/2009).
Among the assayed glycosyl hydrolytic enzymes, only xylanase activity was detected, with maximum values (around 10 and 50 U g^{-1} of d.m. with *P. ostreatus* and *T. versicolor*, respectively) representing lower levels than those previously reported in other studies on fungal SSF. Gawande and Kamat [37] have reported a maximum xylanase activity of 344.5 U g^{-1} by *A. terreus* SSF on wheat bran (supplemented with tryptone or yeast extract). A maximum xylanase production of around 540 U g^{-1} has been achieved by *P. dryinus* and *P. ostreatus* on tree leaves in SSF experiments developed by Elisashvili et al. [25].
The enormous potential of the developed *P. ostreatus* and *T. versicolor* SSF processes on tomato pomace for protease production (Fig. 4) was proved in comparison with data previously reported for other fungi. Even if several SSF processes have been developed for protease production, data for *P. ostreatus* and *T. versicolor* SSF have not been available, so far. On the other hand, many surveys have highlighted the potential of other fungi for the production of proteases by SSF, e.g. *Aspergillus* strains. Villegas et al. [38] have reported a maximum value of 52 U g^{-1} for protease production by *A. niger* SSF on wheat bran after 36 h. When another strain of the same fungus was grown on rice mill wastes, a protease production of 67.7 U g^{-1} has been achieved in the best conditions [39]. Chutmanop et al. [40] have reported a protease production of 1,200 U g^{-1} by *A. oryzae* SSF on a mixture of rice and wheat brans (75: 25, respectively).
When protease production by *P. ostreatus* SSF was compared with that achieved in liquid culture (PDY) with the same strain, similar levels (protease activity ratio to total protein content) were obtained until the 7^{th} day, beginning from which the production in liquid culture increased until it got to values which are 7 fold higher than SSF (Fig.7).

5. Conclusions

The developed processes of *P. ostreatus* and *T. versicolor* SSF on tomato pomace can represent efficient and cost effective systems for enzyme production, requiring low technical and economical inputs. This study showed the good potential of tomato pomace as substrate to produce laccases by *P. ostreatus* and *T. versicolor* SSF, considering that significant enzyme activity levels were achieved without any optimization of culture conditions, neither by nutrient addition nor by O_2 enrichment. Furthermore, the developed processes hold enormous potential for protease production, giving activity levels higher than those reported for the fungi typically considered as the best protease producers such as *Aspergillus* strains.
Moreover, as one of the most significant results of this study, the developed *P. ostreatus* SSF process provides the production of two laccase isoforms not detected in any other liquid culture conditions which have been analysed so far.

Acknowledgments

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Figure Legends

Fig. 1 Time courses of reducing sugar content and of dry weight loss (%) during *P. ostreatus* (**A**) and *T. versicolor* (**B**) SSF on tomato pomace. ■: Weight loss (%); ♦: Reducing sugars.

Fig. 2 Time course of laccase activity production by *P. ostreatus* (**A**) and *T. versicolor* (**B**) SSF on tomato pomace.

Fig. 3 Time course of xylanase activity production by *P. ostreatus* (**A**) and *T. versicolor* (**B**) SSF on tomato pomace.

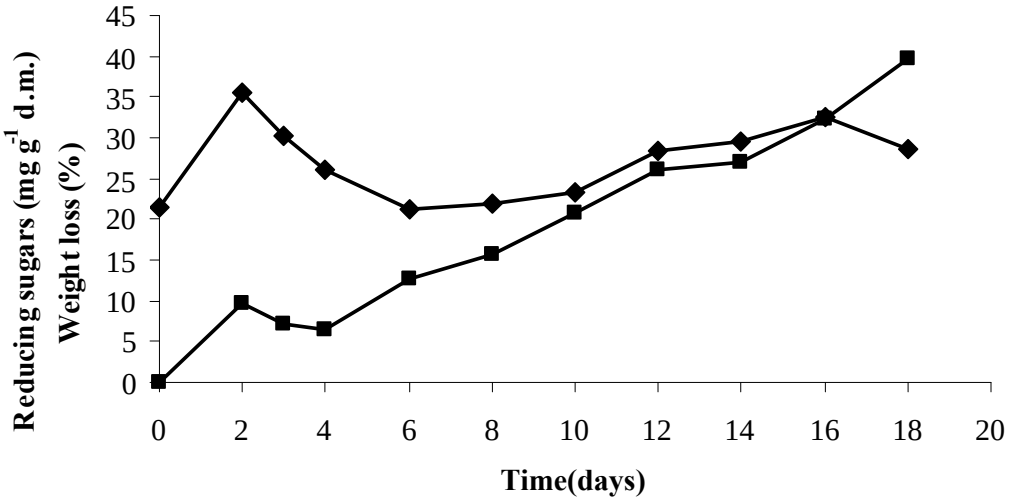
Fig. 4 Time course of protease activity production of *P. ostreatus* (**A**) and *T. versicolor* (**B**) SSF on tomato pomace.

Fig. 5 Production of laccase activity referred to mg of total secreted proteins by *P. ostreatus* in SSF on tomato pomace (■) and in PDY containing liquid culture (♦).

Fig. 6 Native gel for revelation of laccase isoenzymes produced at the 2nd (lane 1), 3rd (lane 2) and 4th (lane 3) of *P. ostreatus* SSF on tomato pomace, compared to those produced in liquid culture in PDY medium supplemented with 150 µM copper sulphate (lane 4). Arrows point to the new bands.

Fig 7 Production of protease activity referred to mg of total secreted proteins by *P. ostreatus* in SSF on tomato pomace (■) and in PDY containing liquid culture (♦).

A


Reducing sugars (mg g-1 d.m.)
Weight loss (%)
Time(days)
0
5
10
15
20
25
30
35
40
45
0
2
4
6
8
10
12
14
16
18
20

B

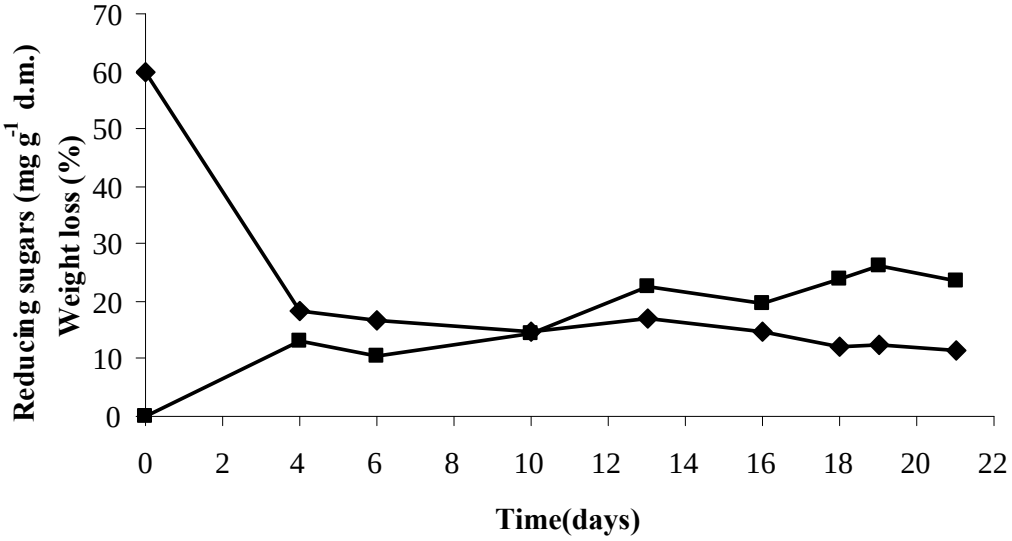

Reducing sugars (mg g-1 d.m.)
Weight loss (%)
Time(days)
0
10
20
30
40
50
60
70
0
2
4
6
8
10
12
14
16
18
20
22

Fig. 1

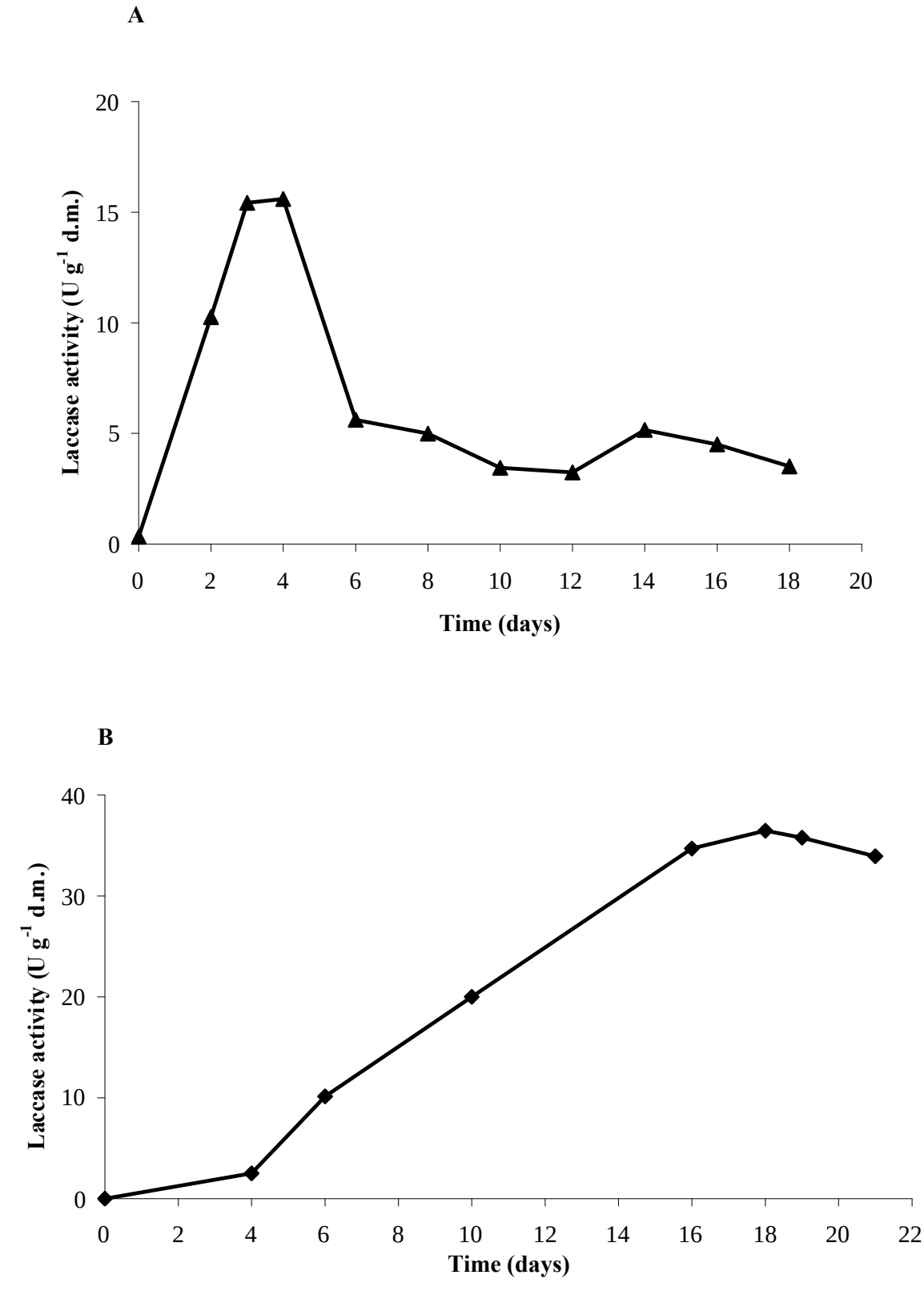

A
Laccase activity (U g-1 d.m.)
0
5
10
15
20
0
2
4
6
8
10
12
14
16
18
20
Time (days)
B
Laccase activity (U g-1 d.m.)
0
10
20
30
40
0
2
4
6
8
10
12
14
16
18
20
22
Time (days)

Fig. 2

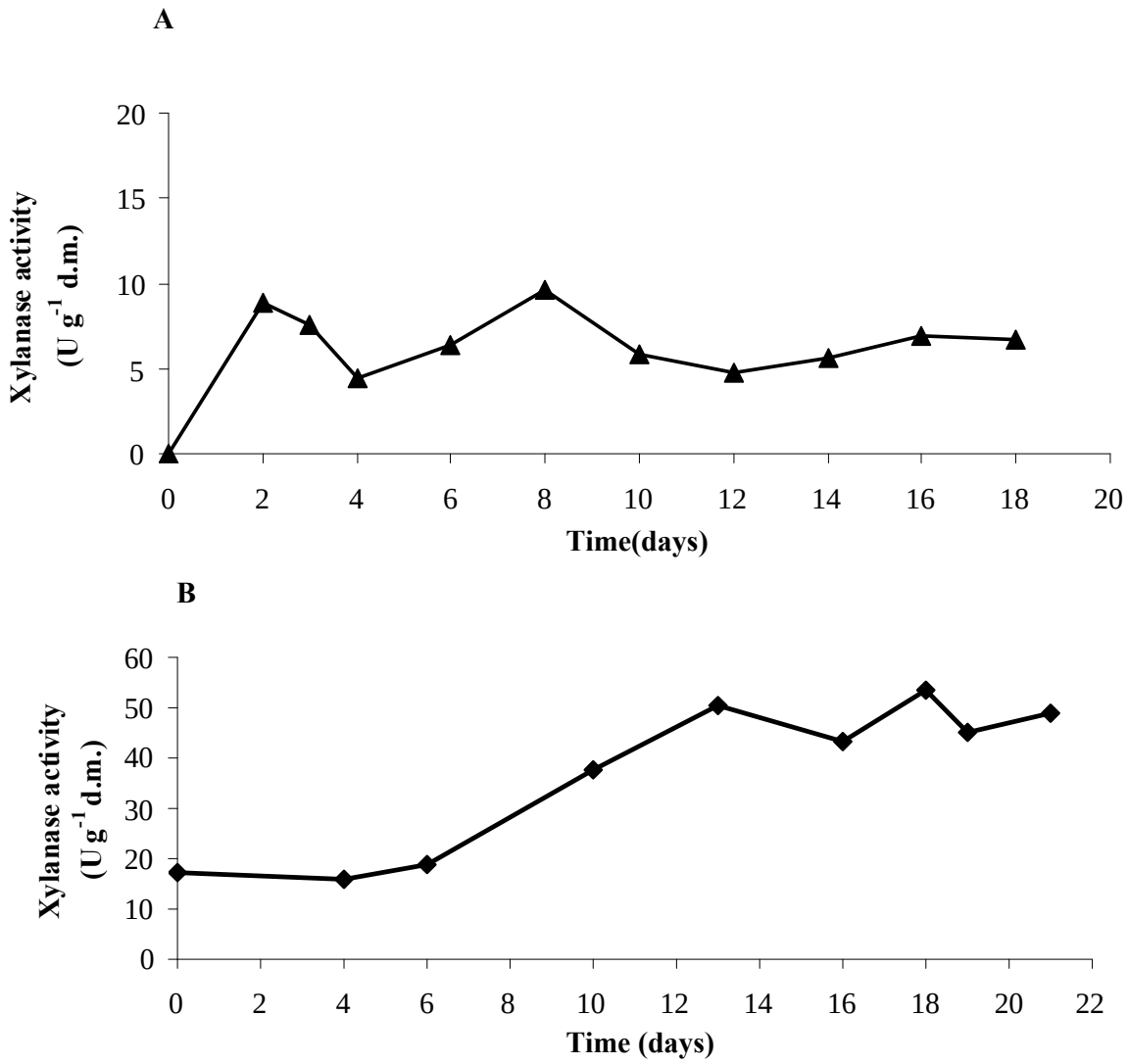
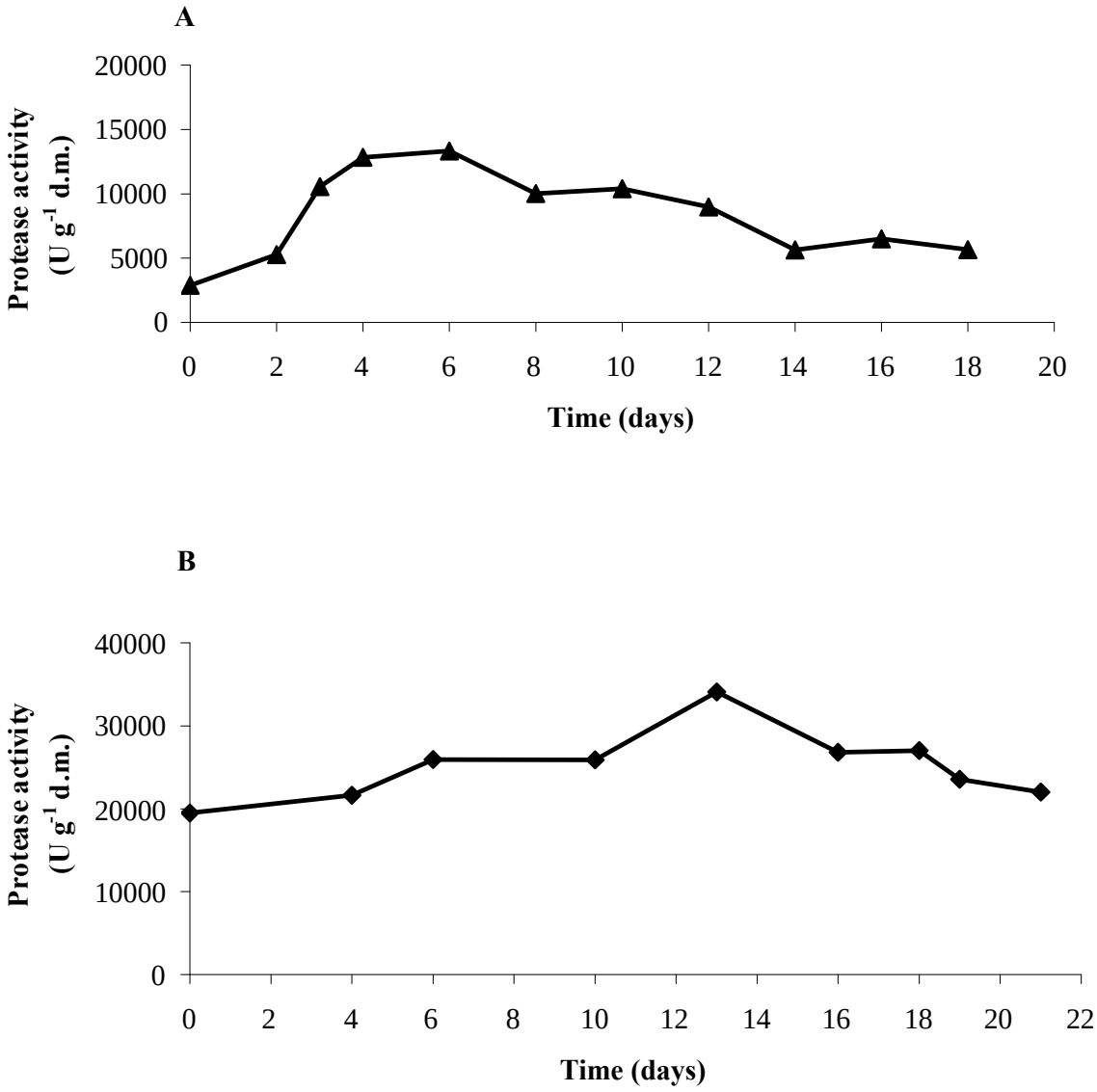

A
Xylanase activity (U g-1 d.m.)
0
5
10
15
20
0
2
4
6
8
10
12
14
16
18
20
Time(days)
B
Xylanase activity (U g-1 d.m.)
0
10
20
30
40
50
60
0
2
4
6
8
10
12
14
16
18
20
22
Time (days)

Fig. 3


A
Protease activity
(U g-1 d.m.)
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15000
10000
5000
0
0
2
4
6
8
10
12
14
16
18
20
Time (days)
B
Protease activity
(U g-1 d.m.)
40000
30000
20000
10000
0
0
2
4
6
8
10
12
14
16
18
20
22
Time (days)

Fig. 4

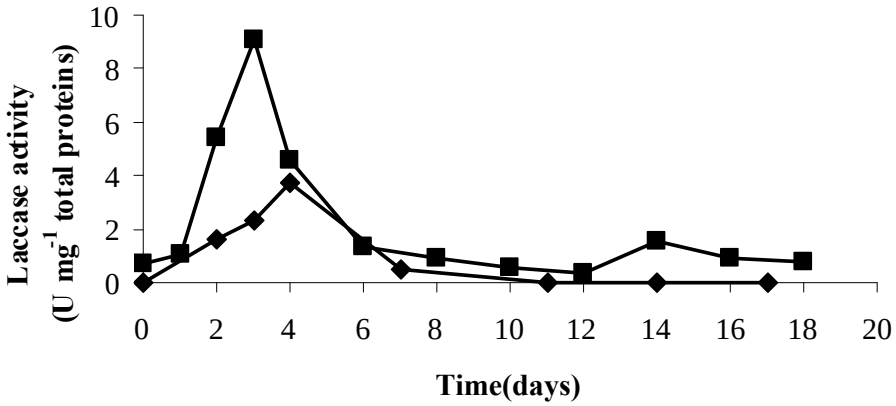

Fig. 5

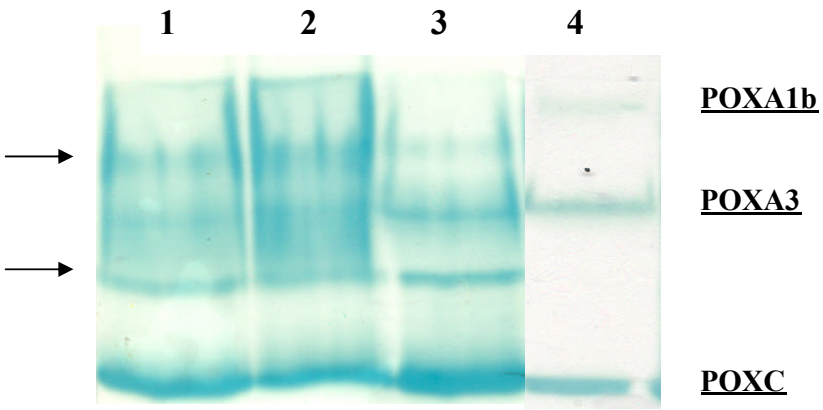

Fig. 6

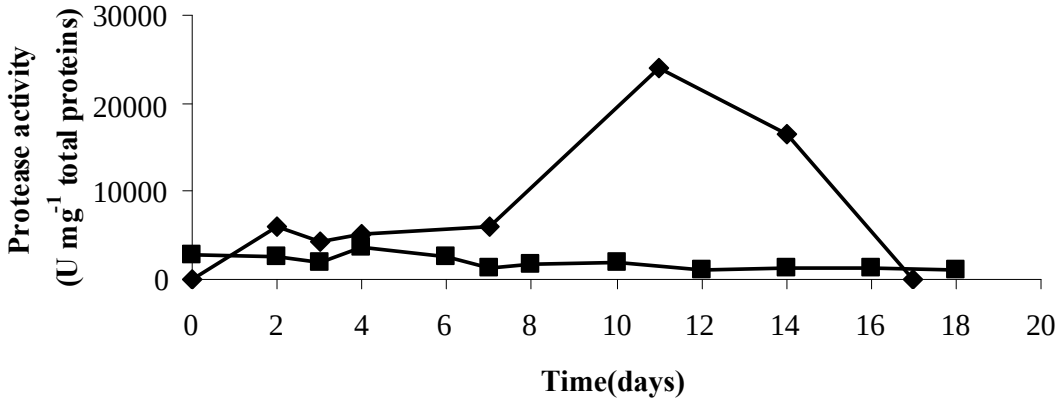

Fig. 7

2.4.2.2 Solid state fermentation for apple pomace valorization

2.4.2.2.1 Introduction

Apples constitute one of the main Italian cultures, with 2 million tons produced in 2006 at national scale, bringing Italy among the main apples producers in Europe and at the fifth place on world scale. Besides, Campania region stands out with around 70.000 t of apples produced in 2006 (Istat, Cultivations, 2008). 5% of collected fruits is transformed, getting to volumes of wastes such as 2.000 t and 70 t (pomace) produced in Italy and in Campania, respectively. These wastes are typically further exploited as fertilizer and as animal fodder. An other alternative way for their upgrading is distillation, for the production of alcoholic beverages, as reported by Rossi from 'Centro Ricerche per le Produzioni animali'. Apple pomace has a content of cellulose of about 23% dry matter, a low level of hemicellulose (6.2% d.m.), and a content of lignin slightly lower than that of cellulose (19.1% d.m.) (Wolter *et al.*, 1980). Moreover, apple pomace has been described as a rich source of polyphenols such as cinnamic acids (caffeic acid), cinnamic acid derivatives (p-coumaric glucoside and chlorogenic acids), flavonols (i.e.: quercitrin, quercetin), flavan-3-ols (catechin, epicatechin and procyanidins). These molecules make it interesting to set up a process in which they are recovered resulting residues containing polysaccharides and all the other nutrients present inside are further exploited (Sanchez-Rabaneda e*t al.*, 2004). Given their composition, these wastes have high potential for being upgraded through both extraction processes and microbial fermentation. As far as the second approach is concerned, several processes have been developed aimed at giving higher value to these wastes, by the obtainment of flavours, feed, food additives or biofuels (Almosnino and Beni, 1991; Joshi and Sandhu, 1996; Stredansky, 1999; Grohamm and Bothast, 1994).
In this research project a process of fungal solid state fermentation has been developed with the two fungi *P. ostreatus* and *T. versicolor*, selected as conversion lignino-cellulolytic microorganisms.

2.4.2.2.2 Materials and methods

Lignocellulosic substrates

Commercially available wheat, employed for fungal pre-culture, was weighted, washed, and moisturized with bi-distilled water (1:1, weight/volume) before autoclaving for 1 hour at 110 °C. After the addition of 1 % (w/w) $CaCO_3$ and of bi-distilled water (1:1, weight/volume), a new sterilization cycle was run.
Apple residues were collected in a local apple processing farm of Campania (Italy). The residues (peels, seeds and stems) were stored frozen. Before sterilizing in autoclave for 1 hour at 110 °C, 1.5 % (w/w) $CaCO_3$ was added in order to get a final pH of 6.0, after sterilization. As for the percentage of humidity, the starting value was kept (71%), without further addition.

Fungal strains

Strains of the white rot fungi *P. ostreatus* (Jacq.:Fr.) Kummer (type: Florida) (ATCC MYA-2306) from ATCC, the Global Bioresource Centre and *T. versicolor* (NBRC4937) from the fungal collection Nite Biological Resource Center (Department of Biotechnology National Institute of Technology and Evaluation, Japan) were used in this study.

The fungi were maintained through periodic transfer at 4 °C on agar (1.5% w/v) plates containing PDY medium [24 g/l potato dextrose (Difco, Detroit, Michigan, USA) and 5 g/l yeast extract (Difco)].

Solid state fermentation

Pre-cultures were prepared by pre-inoculating 100 g of pre-treated wheat in 1 l Erlenmeyer flasks with 5 agar plugs of *P. ostreatus* (11 mm diameter) or 10 agar plugs of *T. versicolor* (9 mm diameter) mycelia, from the edge of a 5-days-old agar culture. After fungal growth in a temperature-controlled incubator at 28°C for six days, the wheat, after mechanical stirring, was used to inoculate 500 g of wet apple pomace prepared as above described. 50 g of the material, made homogeneous by stirring, were transferred in 250 ml flasks, then incubated at 28°C, statically. The fermentation of *P. ostreatus* and *T. versicolor* was followed for 7 days. At suitable time intervals, three flasks were sampled and the content of each flask was used to prepare samples for the analytical determinations. Samples were stored at -80°C, in order to keep enzymatic activities and potentially produced molecules unmodified. Samples aliquots were suspended (1:10 weight/weight) in bi-distilled water for reducing sugars determination and in 50 mM $NaPO_4$ pH 6.8 and added with 1mM phenylmethylsulfonylfluoride (PMSF) for enzymatic assays. When sampes were used for protease activity assays, PMSF was not added to the sodium phosphate total extract. After vigorous mixing by vortex, samples were centrifuged for 30 minutes at 7500 rpm at 4°C (Beckman Coulter, Inc.) and supernatants were subjected to analytical determinations, as below described.

Analytical determinations

Humidity and weight loss evaluation

The percentage of humidity was determined gravimetrically. In a pre-weighed glass plate (W1) 1-2 grams of sampled material (W2) were allowed to dry in a ventilated oven at 65°C for about 24 hours. After cooling in a dessiccator, plates were weighed to get the dry weight (W3). Percentage of humidity was evaluated as {[(W2-W1)-(W3-W1)]/ (W2-W1)} *100. To determine weight loss at each sampling time, the change of total dry matter weight was measured with respect to the beginning of fermentation. Data were reported as percentage of the initial dry matter content.

Analysis of soluble reducing sugar content

Reducing sugars were measured by the 3,5-dinitrosalicylic acid (DNS) method using (100-500 µg) D-glucose as a standard, according to Miller (Miller 1959). Sugar content was assayed on 0.5 ml of the appropriately diluted sample. A DNS solution containing 10 g/l 3,5-dinitrosalicylic acid (Sigma Aldrich), 10 g/l sodium hydroxide, 0.5 g/l anhydrous sodium sulfite and 2g/l phenol was prepared. A 1:1 (volume/volume) mixture of diluted sample and DNS solution was incubated at 95°C for 5 minutes, after mixing by vortex. Mixtures were let to cool to room temperature and, after addition of 3 ml of bi-distilled water, absorbance was measured at 580 nm.

Enzymatic assays

a) Laccase activity

Laccase activity was assayed using 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as a substrate (Giardina *et al*. 1996). The assay mixture contained 2 mM ABTS and 0.1 M sodium citrate buffer, pH 3.0. Oxidation of ABTS was followed by absorbance increase at 420 nm (ε = 36,000 M^{-1} cm^{-1}).

b) Manganese peroxidase

Manganese peroxidase (MnP) activity was determined using manganese sulfate as substrate (Giardina *et al*. 2000). The reaction mixture contained 0.5 mM manganese sulfate and 0.1 mM H_2O_2 in 50 mM sodium malonate buffer, pH 4.5. Oxidation of Mn^{2+} to Mn^{3+} was followed by absorbance increase at 270 nm (ε = 11,590 M^{-1} cm^{-1}) due to the formation of malonate–Mn^{3+} complex.

c) Lignin peroxidase

Lignin peroxidase (LiP) activity was determined using veratryl alcohol as substrate (Tien *et al*. 1984). The reaction mixture contained 2 mM veratryl alcohol and 0.5 mM H_2O_2 in 50 mM sodium tartrate buffer, pH 2.5. Oxidation of veratryl alcohol was followed by measuring the absorbance increase at 310 nm (ε = 9,300 M^{-1} cm^{-1}).

All the enzyme activities were expressed in International Units (IU), where one unit of enzyme activity is defined as the amount of enzyme that oxidizes one µmole of substrate in 1 min.

d) Filter paper activity

Filter paper activity was assayed by a modified version of protocol reported by Ghose (Ghose 1987). A strip of 50 mg (1x 6 cm) of Whatman #1 filter paper was used as a substrate, by incubating it for 1hour at 50°C, in a mixture of 1 ml of 50 mM sodium citrate pH 4.8 and 0.5 ml of the appropriate sample dilution (in 50 mM sodium citrate at pH 4.8). 0.5 ml of this reaction mixture were used to determine the released reducing sugars, by DNS method. A mixture of the analyzed sample and DNS solution (1:1, volume/volume) was incubated at 95°C for 5 minutes. Then, the assay mixture was let cool and, after addition of 4 ml of bi-distilled water, absorbance was measured at 580 nm. Enzyme activity was inferred using d-glucose as a standard.

e) Xylanase activity

Xylanase activity assay was performed according to Bailey *et al.* (21). The reaction mixture consisting of 1.8 ml of a 1.0% (w/v) suspension of birch-wood xylan in 50 mM Na citrate at pH 5.3 and 0.2 ml of enzyme dilution (in 50 mM sodium citrate at pH 5.3) was incubated at 50°C for 5 min. Released reducing sugars were determined by DNS method, by adding 3 ml of DNS solution and then incubating the mixture at 95°C for 5 minutes. Absorbance was measured at 540 nm. One unit of enzyme is defined as the amount of enzyme catalyzing the release of 1 µmol of xylose equivalent per min.

f) Protease activity

The substrate used for protease activity determination is azoalbumin (Sigma-Aldrich). The substrate stock solution (15 mg/ml) was prepared in 3-(N-Morpholino)-propanesulfonic acid (MOPS) buffer 50 mM pH 7. 250 µl of appropriately diluted sample were incubated, in 24 wells plates, with 400 µl of substrate solution for 30 min at 37°C. Reaction was stopped by adding trichloroacetic acid 20% (650 µl). The mixture was centrifuged (600g x 15 min), and 325 µl of NaOH 10M were added to 650 µl of digested substrate. Absorbances were registered at 440 nm, against a blank constituted by substrate and MOPS, in the same proportion used for the reaction. One unit of enzyme is defined as the amount of enzyme catalysing the release of the amount of azodye producing 0.001 unit change in the absorbance read at λ=440 nm).

Evaluation of total protein content

Protein concentration was determined by the method of Lowry *et al.* (1951), using the BioRad Protein Assay (BioRad), with bovine serum albumin as a standard.

Results of the analytical determinations reported in the figures correspond to mean values of three-replicates with a standard deviation lower than 15%, except for protease activity levels for which standard deviation was up to 30%.

Zymogram analyses

Zymogram of laccases

Polyacrylamide gel electrophoresis (PAGE) was performed at an alkaline pH under nondenaturing conditions. The separating and stacking gels contained 9 and 4% acrylamide, respectively. The buffer solution used for the separating gel contained 50 mM Tris-HCl (pH 9.5), and the buffer solution used for the stacking gel contained 18 mM Tris-HCl (pH 7.5). The electrode reservoir solution contained 25 mM Tris and 190 mM glycine (pH 8.4). The gels were stained to visualize laccase activity by using ABTS as the substrate.

Zymogram of xylanases

The zymogram analysis was performed by a modified version of method reported by Ratanakhanokchai ***et al.*** (1999). Samples suspended in $NaPO_4$ buffer (50mM, pH 6.8) were subjected to electrophoresis in an SDS–10% polyacrylamide gel containing 0.1% birchwood xylan (7500.1, Carl Roth). After electrophoresis, the gel was soaked in 25% (v/v) isopropanol by gentle shaking to remove the SDS and renature the proteins in the gel. Then, the gel was washed four times for 30 min at 4°C in 0.1 M acetate buffer (pH 5.5). After further incubation for 60 min at 50°C, the gel was soaked in 0.1% Congo red solution for 30 min at room temperature and washed with 1 M NaCl until excess dye was removed from the active band. After, the gel was submerged in 0.5% acetic acid, the background turned dark blue and the activity bands were observed.

2.4.2.2.3 Results

A process of fungal Solid State fermentation of both *Trametes versicolor* and *Pleurotus ostreatus* on apple pomace was developed, highlighting their ability to fast colonize the substrate. in picture 2.1. The SSF process was characterised by following the changes in different parameters such as humidity and weight loss. This last was used as a measure of substrate colonization (Fig 2.2). Either *T. versicolor* and *P. ostreatus* determined an increasing weight loss up to about 15%, and 32% at the end of fermentation, respectively. Since loss in weight is mainly due to substrate consumption by fungi, to CO_2 evolution, and only to a minor extent to fungal biomass formation, it is exploited as a measure of fungal substrate colonization and growth. The results obtained for the two microorganisms suggest a more extensive substrate colonization for *P. ostreatus* than for *T. versicolor*. During both *T. versicolor* and *P.ostreatus* fermentations, reducing sugars levels were kept costant till the end of fermentation (Fig 2.3). The difference between the starting values of reducing sugars for the two fungi seems to be due to *T. versicolor* metabolism during pre-cultures.

Picture 2.1: Examples of apple pomace colonized by *P. ostreatus* at the 3rd and 8th days of fermentation.

Production of ligninolytic (laccases, LiP and MnP), glycosyl hydrolytic (xylanases and cellulases) and proteolytic enzymes by *P. ostreatus* and *T. versicolor* during SSF on apple pomace was evaluated.

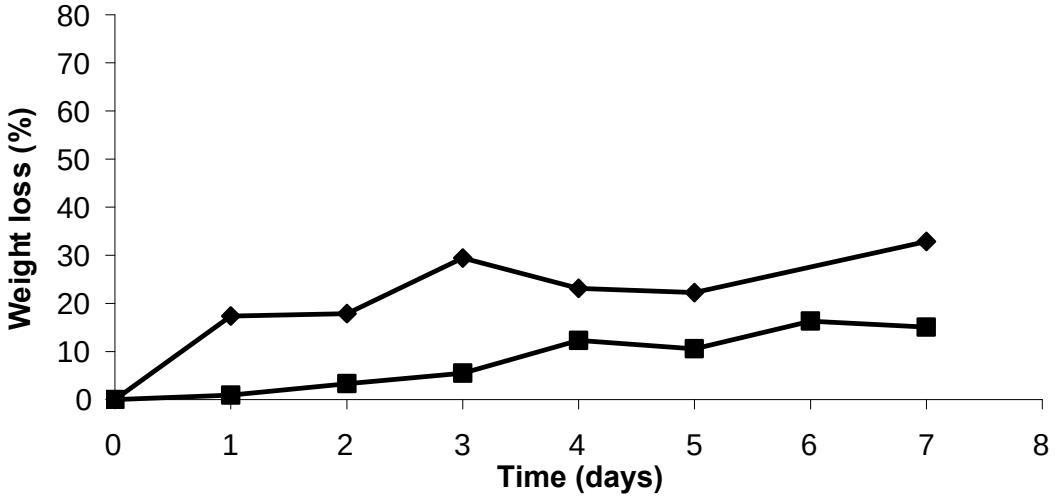

Figure 2.2: Weight loss during *P. ostreatus* and *T. versicolor* apple pomace colonization. *P. ostreatus*: ♦; *T. versicolor*: ■.

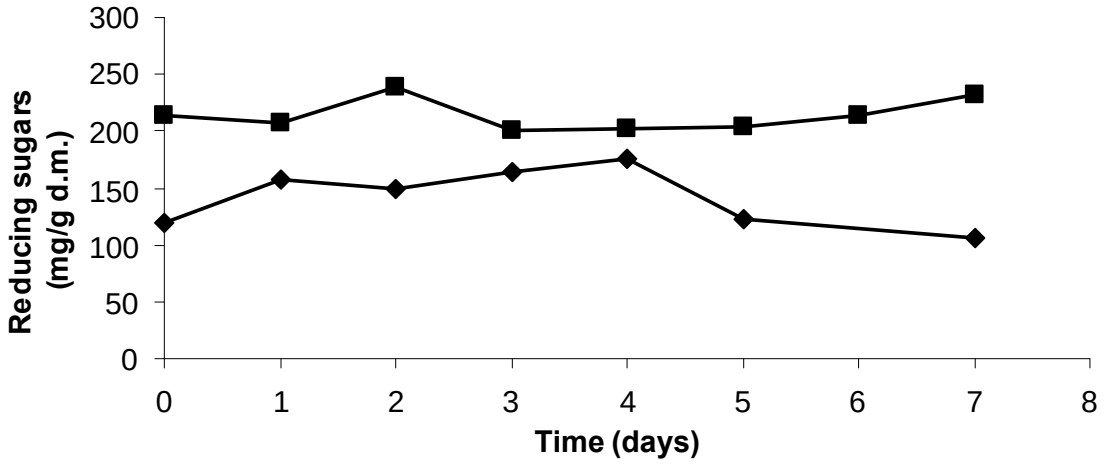

Figure 2.3: Reducing sugars levels during *P. ostreatus* and *T. versicolor* apple pomace colonization, expressed as mg/g d.m.. *P. ostreatus*: ♦; *T. versicolor*: ■.

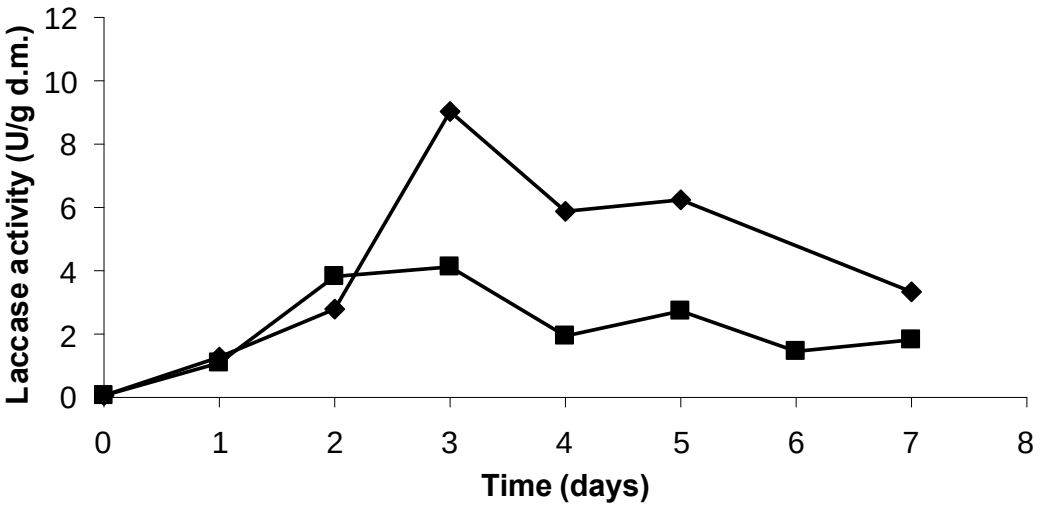

Figure 2.4: Laccase activity levels during *P. ostreatus* and *T. versicolor* apple pomace colonization, expressed as U/g d.m.. *P. ostreatus*: ♦; *T. versicolor*: ■.

As far as laccase activity levels are concerned, maximum values (4 U/g d.m. and 9 U/g d.m., respectively) were reached with *T. versicolor* and *P.ostreatus* at the 3rd day of fermentation (Fig 2.4). Production levels obtained for *P. ostreatus* were compared both in solid and liquid culture (PDY) (Fig. 2.5). Until the end of fermentation, apple pomace SSF was shown to be a better system for the production of high titers of laccases, also because of the low protein concentration of total extract, getting to a value of around 9 U/mg of secreted proteins at the 3rd day.

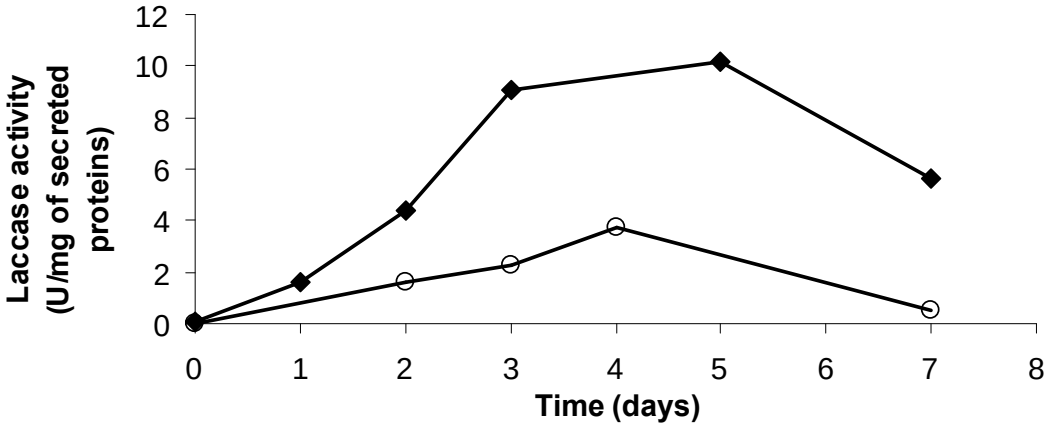

Figure 2.5: Comparison between two different culture conditions of *P. ostreatus*, such as liquid culture (PDY) (○) and SSF on apple pomace (♦).

Secreted laccase isoforms were characterized for both fungi as shown in figure 2.6. The native PAGE analysis of laccase isoenzymes produced by *P. ostreatus* during SSF revealed two more bands besides the three isoenzymes (POXA1b, POXA3, POXC) normally secreted in liquid cultures (Giardina *et al.* 1996; Palmieri *et al.* 1997; Giardina *et al.* 1999; Palmieri *et al*. 2000; Palmieri *et al.* 2003). In the same

experimental conditions, *T. versicolor* produced only one of the two laccase isoforms reported in literature (Bourbonnais *et al.*, 1995).

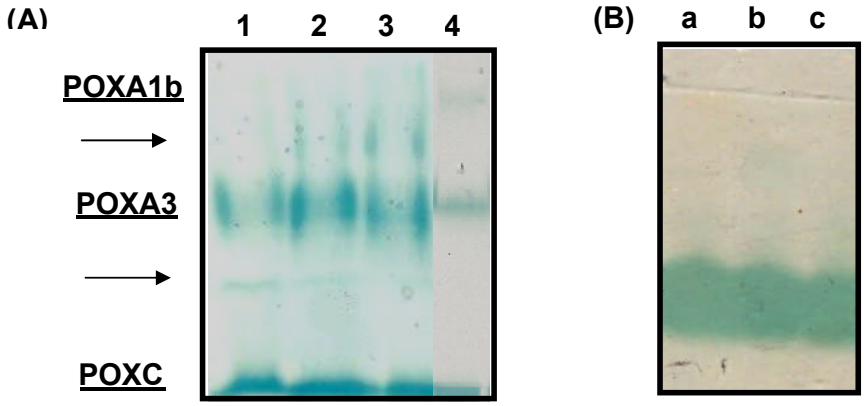

Figure 2.6: Time-course of laccase isoenzymes produced during *P.ostreatus* and *T. versicolor* growth on apple pomace. In panel **A**: Lane 1: 2nd day, Lane 2: 3rd day, Lane 3: 4th day, Lane 4: liquid culture (PDY). Arrows indicate the hypothetical new laccase isoforms. In panel **B**: lane a, b and c stands for *T. versicolor* 2nd, 3rd and 4th days of fermentation.

As far as peroxidases production is concerned, only *T. versicolor* secreted perceptible levels of MnP activity, with a maximum value of 0.89 U/g d.m. attained at the 5th day of fermentation, as reported in fig. 2.7.

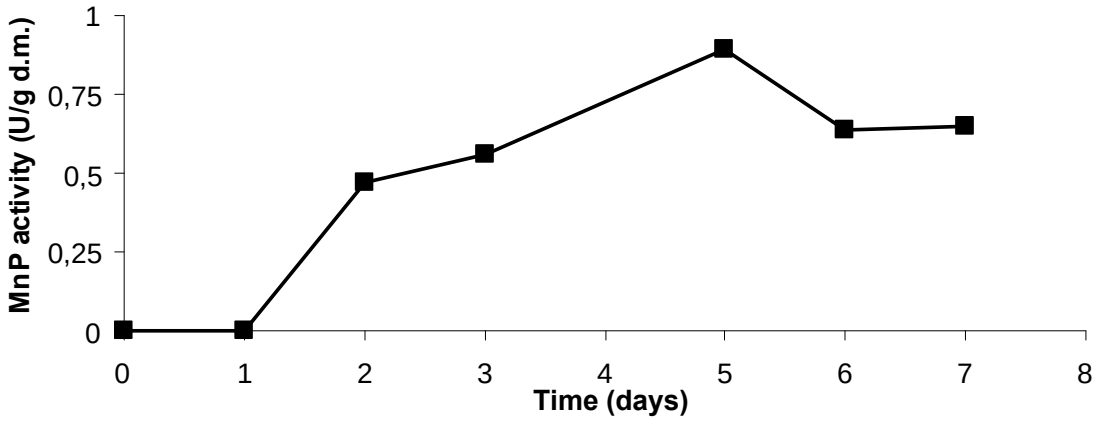

Figure 2.7: MnP activity levels by *T. versicolor*.

Neither *P.ostreatus* nor *T. versicolor* produced appreciable values of LiP in the applied conditions.
Among the investigated glicosyl hydrolases (Filter paper activity -for the estimation of both endo- and exo-cleaving cellulases- and xylanases) only xylanolytic enzymes were produced at detectable levels by both fungi (Fig. 2.8). Indeed, for *T. versicolor* the maximum value of about 35 U/g d.m. of xylanases was obtained at the 1st day of fermentation, later on xylanase levels kept oscillating around this starting value. *P. ostreatus* secreted even higher values of these enzymes than *T. versicolor*, getting to

a maximum of 80 U/g d.m. at the 4th day. The adopted fermentation conditions, did not allow us to detect measurable amounts of cellulases (FPA) for neither fungi.

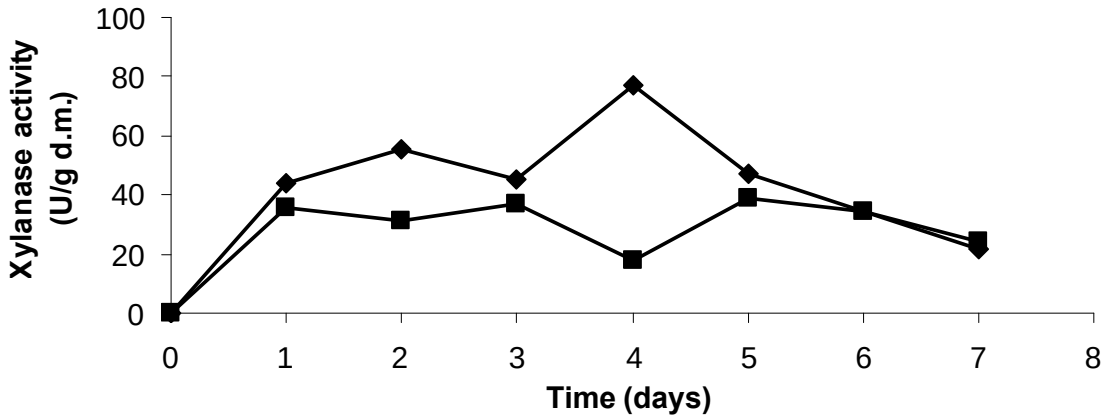

Figure 2.8: Xylanase activity levels during *P. ostreatus* and *T. versicolor* apple pomace colonization, expressed as U/g d.m.. *P. ostreatus*: ♦; *T. versicolor*: ■.

Xylanolytic isoforms produced by both fungi during apple pomace colonization were investigated. Unfortunatly, *P. ostreatus* SSF, it was not possibile to detect active xylanolytic activity through xylanase zymography. This effect could be due to the inactivation of the xylanases in the sample during the handlings before loading.
Xylanolytic isoenzymes profile of *T. versicolor* on apple pomace is reported in figure 2.9.

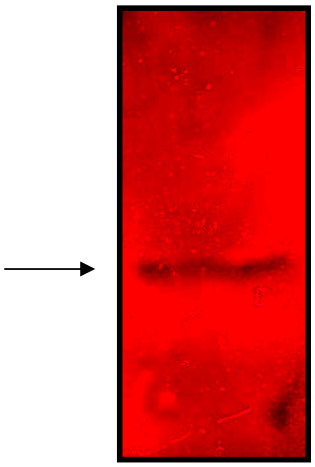

Figure 2.9: Zymogram analysis of xylanolytic enzymes of *T. versicolor* on apple pomace at the 5th day of fermentation.

As for protease activity levels, low levels were attained for both fungi, as foreseeable given the low proteic content of apple pomace (about 4% w/w) (Fig 2.10). Different time courses of protease secretion were observed for *T. versicolor* and *P. ostreatus*. The former secreted about 20,000 U/g d.m. at the beginning of the fermentation, probably produced during its growth on wheat during the previous six days. From the beginning till the end of fermentation protease levels decrease continously.

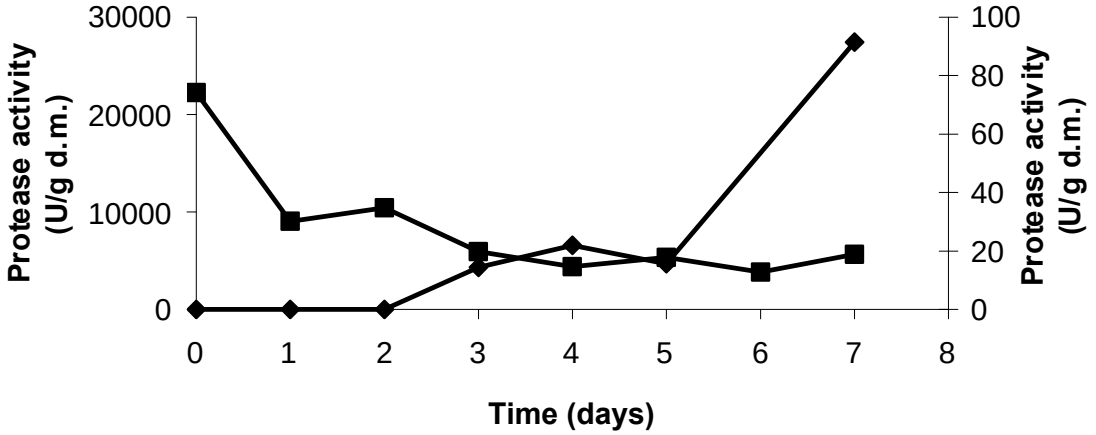

Figure 2.10: Protease activity levels during *P. ostreatus* and *T. versicolor* apple pomace colonization, expressed as U/g d.m.. *P. ostreatus*: ♦; *T. versicolor*: ■.

On the other hand, *P. ostreatus* produced lower levels of proteolytic activities than *T. versicolor* till the 4th day, even though there is an induction of these hydrolytic activities from the 5th day onwards.
Protease production by *P. ostreatus* in solid state fermentation was compared with the production achieved in liquid culture (Fig. 2.11). Proteases specific activity obtained during SSF process on apple waste was very low in comparison to data from liquid cultures.

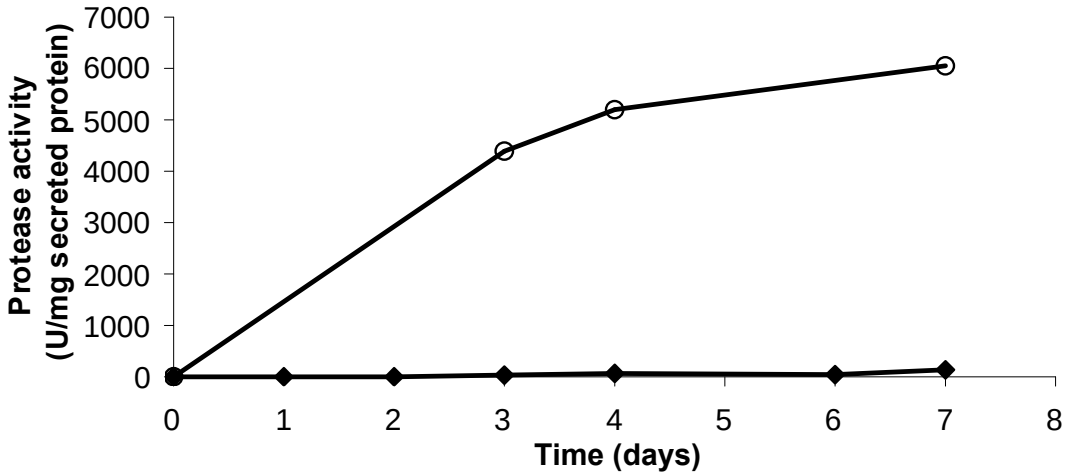

Figure 2.11: Comparison between *P. ostreatus* protease production levels during SSF on apple pomace (♦) and liquid culture (PDY) (○).

2.4.2.2.4 Conclusions

Process parameters, allowing fast and extensive apple pomace colonization, were defined for both *P. ostreatus* and *T. versicolor* on apple pomace. No relevant changes in sugar concentration were measured for both fungi. As far as enzymatic activites are concerned, *P. ostreatus* secreted a maximum of about 9 U/g d.m. of laccase activity at the 3rd day, and it was shown to secrete two more laccase

isoforms than those so far revealed in the analysed liquid cultures. Furthermore, apple pomace SSF was shown to allow better performances, than liquid culture, for the achievement of high titers of laccases. *T. versicolor* secreted lower levels of laccase activities, but it was shown to be able to produce MnP activity, even if at low levels. No Lignin peroxidase activity was measured for both fungi. Apple pomace was useful to achieve high levels of xylanases, at the beginning of the fermentation, *P. ostreatus* secreting higher levels than *T. versicolor*. Low levels of protease activity were obtained, for both microorganisms.

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2.5. Identification of xylanolytic enzymes from *Pleurotus ostreatus*

2.5.1 Introduction

Among the different enzymatic activities taken into account, xylanases have potential applications in different fields, such as pulp and paper, food, beverages and feed industries. Besides, to the best of our knowledge, there is no report in the literature on the purification of xylanases from *P. ostreatus*.

Results previously reported in this study about the achieved values of xylanase activity (Section 2.4), as well as the wide range of market applications of these enzymes led us to further investigate produced xylanases for their indentification and characterization. Thus, trials for setting up a purification strategy were performed.

2.5.2 Materials and Methods

Extraction of soluble enzymes for xylanase purification

Moist material, made up by tomato pomace colonized by *P. ostreatus*, was suspended (1:10 w/w) in $NaPO_4$ 50 mM pH 6.8 in Beckman jars in agitation for 1 h (150 rpm). PMSF 1mM was added and the obtained supernatant was used for further analyses.

Evaluation of total protein

Protein concentration was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

SDS-PAGE

Proteins were separated on sodium dodecyl sulfate (SDS)-PAGE gels as described by Laemmli (1970).

Xylanase activity

Xylanase activity assay was performed according to Bailey *et al.* (21). The reaction mixture consisting of 1.8 ml of a 1.0% (w/v) suspension of birch-wood xylan in 50 mM Na citrate at pH 5.3 and 0.2 ml of enzyme dilution (in 50 mM sodium citrate at pH 5.3) was incubated at 50°C for 5 min. Released reducing sugars were determined by DNS method, by adding 3 ml of DNS solution and then incubating the mixture at 95°C for 5 minutes. Absorbance was measured at 540 nm. One unit of enzyme is defined as the amount of enzyme catalyzing the release of 1 µmol of xylose equivalent per min.

Zymogram of xylanases

The zymogram analysis was performed by a modified version of method reported by Ratanakhanokchai *et al.* (1999). Samples suspended in $NaPO_4$ buffer (50mM, pH 6.8) were subjected to electrophoresis in an SDS–10% polyacrylamide gel containing 0.1% birchwood xylan (7500.1, Carl Roth). After electrophoresis, the gel was soaked in 25% (v/v) isopropanol by gentle shaking to remove the SDS and renature the proteins in the gel. Then, the gel was washed four times for 30 min at 4°C in 0.1 M acetate buffer (pH 5.5). After further incubation for 60 min at 50°C, the gel was soaked in 0.1% Congo red solution for 30 min at room temperature and washed with 1 M NaCl until excess dye was removed from the active band. After, the gel was submerged in 0.5% acetic acid, the background turned dark blue and the activity bands were observed.

Ammonium sulphate precipitation
Clear supernatant was treated with ammonium sulphate at 80% of saturation. Addition of ammonium sulphate was carried out during continuous stirring of the total extract at 4°C. The solution was kept stirring at 4°C overnight. The precipitated proteins were, then, removed by centrifugation at 6500 rpm for 45 minutes at 4°C. The pellet so far obtained was dissolved in $NaPO_4$ 50mM pH 7.

Hydrophobic interaction chromatography (HIC)
The sample obtained after the ammonium sulphate
precipitation step was subjected to a hydrophobic interaction chromatography with the process parameters reported in table 2.19.

COLUMN	Phenyl Sepharose (Pharmacia, Sweden) Column volume (Cv): 5 ml
FLOW RATE	1 ml/ min
BUFFER A	$NaPO_4$ 50 mM, $(NH_4)_2SO_4$ 1M, pH 7
BUFFER B	$NaPO_4$ 50 mM, pH 7
ELUTION	0-100% B (7 Cv), 100% B (3 Cv)

Table 2.19: Hydrophobic interaction chromatography parameters.

The column was connected to an AKTA purifier chromatographic system, coupled with the UNICORN software. The enzyme was eluted using $NaPO_4$ 50 mM pH 7 at a flow rate of 1 ml/min. Fractions of 2 ml were collected and used for the next chromatography.

Ultrafiltration
The excess of salts present in the sample coming from HIC chromatography, were removed by stirring it in a 50-ml ultrafiltration cell (AMICON) using polyethersulfone membrane (cut off: 10 kDa), adding $NaPO_4$ 50 mM pH 7.

Anion exchange chromatography
Before loading the sample, the column (AKTA purifier chromatographic system, coupled with the UNICORN software) was equilibrated with $NaPO_4$ 50 mM pH 6.8. This chromatografic step was run with the process parameters reported in table 2.20. The unbound proteins were eluted in $NaPO_4$ 50 mM pH 6.8, whereas the bound proteins were eluted with a linear gradient of NaCl (0-1M) in the same buffer. Flow rate was adjusted at 5 ml/min and fractions of 2 ml were collected.

COLUMN	DEAE HiTRAP FF (Pharmacia, Sweden) Column volume (Cv) = 5ml
FLOW RATE	5 ml/min
BUFFER A	$NaPO_4$ 50 mM pH 6.8
BUFFER B	$NaPO_4$ 50 mM NaCl 1M pH 6.8
ELUTION	0-100% B (20 Cv)

Table 2.20: Anionic exchange chromatography parameters

2.5.3 Results

2.5.3.1 Analyses of *P. ostreatus* genome for the presence of xylanases

As shown in table 2.21, *P. ostreatus* genome contains at least 5 genes coding for xylanases. Two xylanases from *P. ostreatus* show the highest similarity with the xylanase from *Schizophyllum commune* that has been fully characterized (Oku *et al.*, 1993). Xylanases from *P. ostreatus* belong to both Glycoside Hydrolase family 10 (GH10) and family 11 (GH11). These two families have been established on the basis of sequence similarities, tridimensional structures and hydrophobic cluster analysis. Different kinds of glycosilases belong to GH10 such as endo-1,4-β-xylanases (EC 3.2.1.8), endo-1,3-β-xylanases (EC 3.2.1.32) and cellobiohydrolases (EC 3.2.1.91). The proteins belonging to this family are characterised by a high molecular mass and a low pI, they display an $(\alpha/\beta)_8$ barrel fold and an active site with the shape of a shallow groove. Studies on substrate specificity of GH10 endo-1,4-β-xylanases have revealed that they may not be entirely specific for xylan, being active also on low molecular mass cellulose substrates. On the other hand, enzymes belonging to GH11 family are generally characterized by a low molecular weight, a high pI, a β-jelly roll fold structure and an active site similar to a deep cleft. Differently from xylanases belonging to family 10, these enzymes are active exclusively on D-xylose containing substrates. They have a lower catalytic versatility than family 10 xylanases and indeed the products of their action can be further hydrolyzed by the family 10 enzymes (Collins *et al.*, 2005). Moreover, xylanases can show carbohydrate binding domains (CBDs), promoting the association of the enzyme to the substrate, thus facilitating the access to the target glycosidic bonds. The signal peptides (SP) are the signals for xylanases sorting in the extracellular medium.

Gene	Annotated domains	Notes
e_gw1.8.1289.1 aa: 331 Protein ID: 33244 Location: PleosPC15_1/scaffold_8:1998789-2002390	SP GH 10	64% identity with Xylanase (*A. bisporus*)
fgenesh2_pg.8_#_527 aa: 411 Protein ID: 170518 Location: PleosPC15_1/scaffold_8:2002807-2004517	SP GH 10 CBD 1	55% identity xylanase A (*P. chrysosporium*) 62% identity with Xylanase (*A. bisporus*)
estExt_fgenesh2_pg.C_100102 aa: 295 Protein ID: 177152 Location: PleosPC15_1/scaffold_10:445032-4601	SP GH 11 CBD 1	44% identity with hypothetical xylanase (*N. crassa* OR74A) 76% identity with xylanase (*S. commune*)
estExt_genemark.C_10290 aa: 389 Protein ID: 172292 Location: PleosPC15_1/scaffold_1:833105-835289	SP GH 10 CBD 1	48% identity with xylanase (*V. volvacea*)
fgenesh1_pm.C_scaffold_10000056 aa: 234 Protein ID: 52897 Location: PleosPC15_1/scaffold_10:440741-442948	SP GH 11 Concanavalin A-like lectin	77% identity with xylanase (*S. commune*)

Table 2.21: *P. ostreatus* xylanase genes, **SP**: Signal Peptide, **GH**: Glycoside Hydrolase, **CBD**: Carbohydrate binding domain

2.5.3.2 Xylanolytic isoenzymes produced during tomato Solid State Fermentation.

Zymogram analyses revealed that *P. ostreatus*, during its growth on tomato pomace, produces two different xylanolytic isoenzymes at the 2nd and 7th days of SSF (figure 2.11).

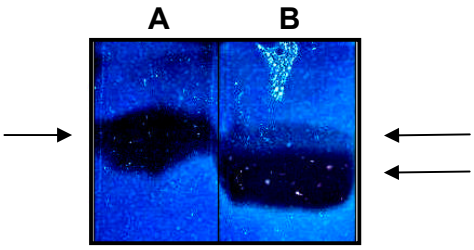


Figure 2.11: Zymogram of *P. ostreatus* xylanases, during SSF on tomato pomace. Lane A: 2nd day of fermentation; lane B: 7th days of fermentation.

The two isoenzymes differ in their electrophoretic mobilities and are expressed to a different extent at the two times, as shown in figure 2.11. The results achieved so far and reported in this study and the information coming from the analysis of *P. ostreatus* genome led us to further investigate xylanases for their identification and characterization.

2.5.3.3 Enrichment of xylanases from *P. ostreatus*

Crude protein extract from a preparative solid state fermentation on tomato waste (seventh day) was subjected to a three-step purification scheme involving ammonium sulphate precipitation [80% $(NH_4)_2SO_4$], hydrophobic interaction chromatography (HIC), as second step, and anion-exchange chromatography, as the final step.
The first-step of proteins salting out with ammonium sulphate is meant for sample concentration and it was chosen since it is really useful when working with high volumes. Afterwards, hydrophobic interaction chromatography was chosen to remove salts from the sampe to be loaded, in alternative to conventional dialysis procedures. The dialysis is usually performed in tubes, commonly made of regenerated cellulose. However, we verified a decrease in xylanase activity following overnight dialysis in the above mentioned dialysis tubes. This phenomenon was ascribed to the presence of the carbohydrate binding domain (CBD) in the investigated enzymes. Table 2.22 summarizes data from the analyses performed in this study, demonstrating that xylanase activity yield decreased by about 50% after overnight dialysis.

Sample	Activity	Volume	Total Activity	Yield
	(U/ml)	(ml)	(U_{tot})	(%)
Before dialysis	0.66	20	13.2	100
After dialysis (2h)	0.55	20	10.9	83
After dialysis (overnight)	0.30	20	6	46

Table 2.22: Dialysis trials on the crude protein extract

The yield of the procedures for xylanases enrichment, adopted in this work, are reported in table 2.23. The elution profile of the hydrophobic interaction chromatography is reported in figure 2.12, where it is possible to see two protein peaks, both showing xylanase activity. Sample before and after hydrophobic chromatography were analysed by SDS-PAGE, to evaluate the efficiency of the purification step (Fig. 2.13).

STEP	Volume	Proteic concentration	Activity	Total Activity	Specific activity	Yield
	(ml)	(mg/ml)	(U/ml)	(U_{tot})	(U/mg)	(%)
Protein extract after thawing	160	0.13	0.34	54.44	2.63	100
I STEP $(NH_4)_2SO_4$ precipitation	25	0.31	0.42	10.43	1.32	19
II STEP Hydrophobic interaction	14	0.07	0.29	4	4.43	7
III STEP Anion exchange	9	0.016	0.33	2.97	20.62	5.4

Table 2.23: Scheme of xylanases enrichment from *P. ostreatus*.

The fractions corresponding to the two peaks of xylanase activity were pooled, and after concentration and salts removal by ultrafiltration, the sample was loaded on a DEAE Sepharose column. The elution profile obtained during DEAE chromatography is shown in figure 2.14. Xylanase activity was detected in the fractions which poorly bound the DEAE column. Samples from DEAE were subjected to SDS-PAGE, as shown in figure 2.15.

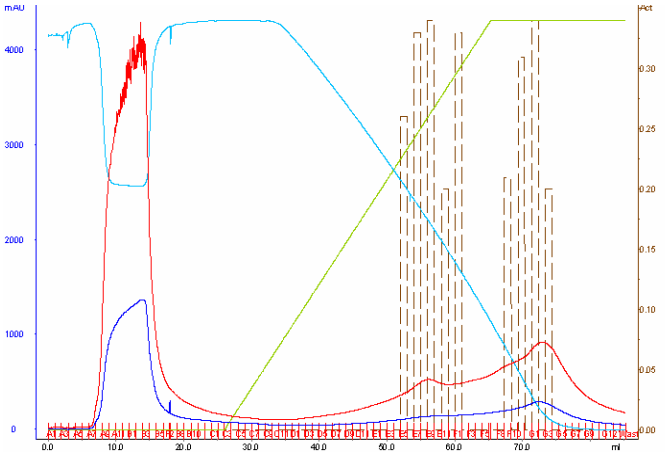
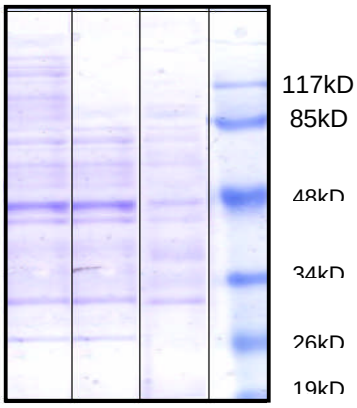


Figure 2.12: Elution profile of the Hydrophobic interaction chromatography (HIC). X axis represent the volume of buffer B. Blue: Abs 280nm, Red: Abs 220nm, Azure-blue: Conductivity (%), Green: Concentration elution buffer (%), Brown: Xylanase activity (U/mL)



Lane A: Crude proteic extract
Lane B: Ammonium sulphate precipitate
Lane C: HIC
Lane D: Markers

Figure 2.13: SDS-PAGE analyses after both ammonium sulphate precipitation and HIC. The same amount (10 micrograms total proteins) for each sample was loaded on the polyacrylamide gel.

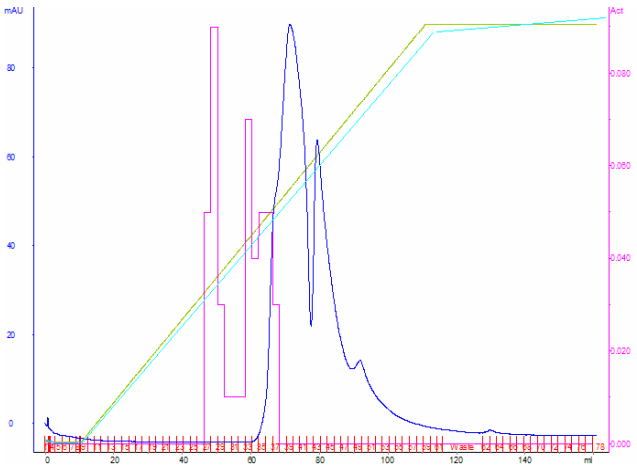
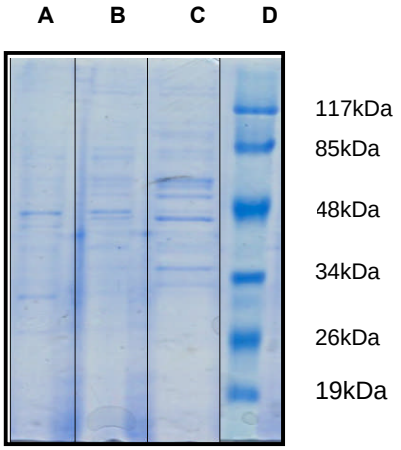


Figure 2.14: Elution profile of the anion exchange chromatography (DEAE). Blue: Abs 280nm, Azure-blue: Conductivity (%), Green: Concentration elution buffer, Pink: Xylanase activity (U/mL)



Lane A: HIC
Lane B: sample from HIC after being concentrated
Lane C: Anion exchange
Lane D: Markers

Figure 2.15: SDS-PAGE analyses of after both HIC and DEAE chromatography. The same amount (10 microgram total proteins) of each sample was loaded on the polyacrylamide gel.

After the three steps of purification xylanase activity was enriched about 8 fold, with a final yield of 5,4 %. About 80% of xylanase activity was lost during ammonium sulphate precipitation. Thus, this step constitutes a bottleneck for the whole purification strategy, that should be overcome. The most active sample coming from the DEAE chomatography was analysed trough proteomic techniques. Four bands among the more aboundant on the SDS-PAGE were recovered from the gel (Fig. 2.16), and were hydrolysed with trypsin. The peptides thus obtained were loaded on a Q-TOF spectrometre. The values of mass to charge ratios of the different peptides were, then, submitted to the analysis of a Mascot search engine, exploiting the availability of the annotated genome of *P. ostreatus*.

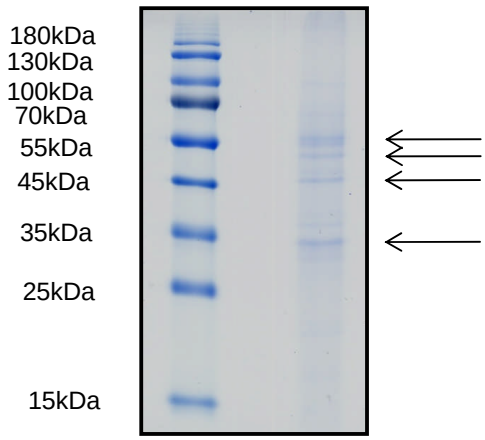


Figure 2.16: SDS-PAGE of the sample coming from DEAE chromatografy, that was analysed through mass spectrometry techniques. Arrows point at the bands that were taken into account.

This procedure allowed us to identify a protein involved in the metabolism of plant cell wall polysaccharides, with the sequence corresponding to an α-galactosidase (De Vries *et al.*, 1999). As inferable from literature data, this enzyme is involved in arabinoxylans hydrolysis (galactose+ xylose/ arabinose) and carries out an ancillary function in hemicellulose hydrolysis, freeing the main chain galactomannans from galactose of the side chain.
α-Galactosidase has been found several industrial applications. Zeilinger *et al.* (1993) reported the use of this enzyme in the paper industry, whilst Szendefy *et al.* (2006) applayed it in the hydrolysis of galactomannose, one of the main wood hemicellulose components. α-Galactosidase has been used in the sugar beet refining, in the improvement of gellifying properties of thickners, and galacto-oligosaccharide reduction in soybean milk and vegetable products (Ademark *et al.*, 2001).
However, it remains to be further investigated the xylanolytic activities visualised on the native PAGE probably responsible for the hydrolysis of the xylan main chain.

2.5.4 References

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- **Zeilinger, S., Kristufek, D., Arisan-Atac, I., Hodits, R., Kubicek, C.,** Conditions of formation, purification and characterization of an α-galactosidase of *Trichoderma Reesei* RUT 30. *Appl. Environ. Microbiol*. (1993): 1347-1353.

3. Conclusions

In the present research thesis, fungal SSF processes for lignocellulosic wastes conversion were developed, aiming at obtaining industrially relevant high added value products.
The first step was the selection of lignocellulosic wastes taking into account volumes and times of production, macromolecular composition and existing alternative uses. Data were collected about different categories of wastes such as the residues coming from herbaceous and woody cultures, food processing factories, the green and organic fractions of municipal solid wastes. Usually, the residues from herbaceous and woody cultures have as main drawback the fact that they are left on the ground to produce humus: process of crucial importance for soil fertility maintenance. Thus, they were not selected for the subsequent process development. As far as food farming residues are concerned, the presence of factories with the problem of wastes disposal highlights the importance of finding alternative ways for their exploitation, being landfilling, burning or animal feeding the commonly adopted ways for their disposal. These strategies, indeed, have both energetic and environmental drawbacks.
Tomato and apple pomaces were selected as starting raw materials. Tomato residues were chosen because of the high amounts yearly produced, and the need of tomato processing industries to find alternative options for their disposal. On the other hand, apple pomace was chosen, even if not produced at the same levels as tomato processing residues, being regarded as exploitable mainly for its composition and presence of valuable molecules with high market profitability.
As the second step of this research thesis, available literature data on fungal conversion abilities towards the different polymeric components of wastes were analysed. Thus, a comprehensive result of this work was the setting up of an internal data bank of fungi classified according to their prevalent conversion abilities. To this purpose, data on compositional changes and on levels of enzymatic activities were taken into account. As far as the conversion performances are concerned, *T. versicolor* NBRC (IFO) 4937 stands out as both cellulolytic and ligninolytic fungus, *P. tuber-regium* can be selected as hemicellulose converter, whilst *Phellinus pini* results the best lignin degrader. The panel of selected microorganisms was further enlarged, taking into account the enzymatic activities involved in lignocellulose conversion. *T. hirsuta* stands out as producer of laccase activity, whereas *Moniliella* spSB9 results the best fungus for pectin conversion. *P. ostreatus* 2191 appears the best endoglucanase producer, while *P. ostreatus* 2175 produces the highest levels both of xylanase and filter paper activities. *Aspergillus niger* BTL and *Neurospora crassa* DSM1129, intriguingly, secrete high amounts of different enzymes such as polygalacturonases, pectinases, endoglucanases and xylanases, thus resulting useful for the conversion of different polysaccharidic fractions at the same time. As well, *P. dryinus* IBB903, secreting high amounts of different classes of glycosyl hydrolytic enzymes, could be used for the concomitant conversion of the polysaccharidic components of ligno-cellulose. It is worth noting that fungal performances in lignocellulose conversion are strongly influenced by the exploited substrate, thus is not possible to strictly forsee their performances on different materials. Furthermore, it seems of high relevance the high methabolic adaptability shown by fungi belonging to *Pleurotus* genus, as reported in the analysed data.
For the development of solid state fermentations on apple and tomato processing residues, *P. ostreatus* and *T. versicolor* were chosen as conversion microorganisms. Culture conditions were set up, allowing tomato pomace colonization and transformation. This study showed the good potential of tomato waste as substrate

for laccases production by *P. ostreatus and T. versicolor* SSF, considering that significant enzyme activity levels were achieved without any optimization of culture conditions, neither by nutrient addition nor by O_2 enrichment. Furthermore, SSFs on tomato pomace hold enormous potential for protease production, allowing the achievement of activity levels higher than those reported for fungi typically exploited for protease production, such as those belonging to the *Aspergillus* genus. Furthermore, a process of fungal SSF was developed on apple pomace, identifying the parameters (pH, humidity) allowing fast substrate colonization by both fungi. It was shown that apple pomace induced high levels of xylanases, with *P. ostreatus* secreting higher levels than *T. versicolor*. However, both fungi secreted levels of laccase activities that are lower or comparable with those achieved during tomato pomace colonization. *T. versicolor* produced low levels of Manganese peroxidase. On this substrate, low levels of protease activity were obtained, for both microorganisms.

Furthermore, both tomato and apple pomace SSFs were shown to be better systems than liquid culture for the production of high laccase levels by *P. ostreatus*. Moreover, as one of the most significant results of this study, the developed *P. ostreatus* SSF processes provide the production of two laccase isoforms not detected in any other liquid culture conditions so far analysed.

Finally, a strategy for *P. ostreatus* xylanase enrichment was explored. The first trials for the identification of xylanolytic enzymes, allowed the detection and identification of an α-galactosidase. This enzyme is not involved in the hydrolysis of the xylan backbone, but plays its role in the removal of galactose units from both galactomannans and arabinoxylans, acting as ancillar xylanolytic enzyme. The xylanolytic activities involved in xylan backbone hydrolysis, and visualized by zymogram analysis, have to be further investigated.

ABBREVIATIONS

SSF: Solid State Fermentation
SmF: Submerged Fermentation
PMSF: Phenylmethylsulfonylfluoride
ABTS: 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)
LiP: Lignin Peroxidase
MnP: Manganese Peroxidase
IU: International Unit
d.m.: dry matter
DNS: 3,5-dinitrosalicylic acid
SDS: Sodium Dodecyl Sulphate
PAGE: Polyacrylamide Gel Electrophoresis
HIC: Hydrophobic Interaction Chromatography
SP: Signal Peptide
GH: Glycoside hydrolase
CBD: Carbohydrate Binding Domain

Appendix

Communications, Publications, Courses, Experiences in foreign laboratories.

Communications:

Faraco, V., Iandolo, D., Giardina, P., Spezie, R., Anastasio, M., and Sannia, G.
Design of new bio-processes for conversion of ligno-cellulose wastes into energy and high added value products, First European workshop on biotechnology for lignocellulose biorefineries, Copenhagen, Danemark, March 26-28, 2008

Faraco, V., Iandolo, D., Giardina, P., and Sannia, G.
Nuovi approcci alla valorizzazione della frazione organica dei rifiuti civili: le potenzalità dei funghi nella valorizzazione degli scarti, Ecomondo, Rimini, November, 2008

Faraco, V., Iandolo, D., Giardina, P., Perrault-Gaime, I., Augur, C., and Sannia, G.,
Development of fungal solid state fermentation processes for conversion of ligno-cellulose wastes into energy and high added value products, The Second Annual Workshop of COST FP0602, Enzymatic fiber modification and hydrolysis, Biel, Switzerland December 4-5, 2008

Faraco, V., Iandolo, D., Giardina, P., and Sannia, G.
Design of a novel process for bio-ethanol production from lignocellulosic wastes. Workshop Energymed 07, Napoli 03/2007.

Publications:

Iandolo, D., Giardina, P., Sannia, G. and Faraco, V.
Development of fungal solid state fermentation processes on vegetable wastes, in Proceedings book, ITALIC 5, Science & technology of biomasses, Advances and challenges (2009): 238-241

Iandolo, D., Giardina, P., Sannia, G. and Faraco, V.
High added-value products from ligno-cellulosic wastes through fungal solid state fermentation processes, in Proceedings book, ITALIC 5, Science & technology of biomasses, Advances and challenges (2009): 213-216.

Iandolo, D., Piscitelli, A., Sannia, G. and Faraco, V.
Enzyme production by solid substrate fermentation of *Pleurotus ostreatus* and *Trametes versicolor* on tomato pomace, *submitted to Biochemical Engineering Journal*

Courses:

18/10/2009-23/10/2009: PhD course on Biotechnology for Bioethanol Production, presso il L'Università Chalmers, Department of Chemical and Biological Engineering, Göteborg, Svezia.

Experiences in foreign laboratories:

06-2008-08-2008: stage in the laboratories of *Institut de Recherche pour le développment* (IRD), at the University Paul Cézanne, Marseille (France), under the supervision of Dr. Christopher Augur. The stage was founded by University of Naples Federico II, with the "Programma di scambi internazionali tra l'Università degli Studi di Napoli Federico II ed Istituti di ricerca stranieri per la mobilità di breve durata di docenti, studiosi e ricercatori".

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