PROCESSES FOR BIOBUTANOL PRODUCTION FROM RENEWABLE RESOURCES

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RIASSUNTO

Negli ultimi decenni il riscaldamento globale, l'instabilità di approvvigionamento delle risorse energetiche di origine fossile, e la consapevolezza di limitare l'uso delle risorse naturali hanno catalizzato l'interesse per lo sviluppo di processi per la produzione di energia, ed in particolare di vettori energetici, da fonti rinnovabili. In questo contesto e allo scopo di massimizzare lo sfruttamento delle risorse rinnovabili di origine organica, è stato sviluppato di recente il concetto di bioraffineria: l'articolazione di processi ecosostenibili tesi a sfruttare fonti organiche rinnovabili per la produzione congiunta di biocarburanti, energia e composti chimici. Il concetto di bioraffineria è analogo al concetto di raffineria del petrolio: processi articolati mirati alla massimizzazione dello sfruttamento del petrolio per produrre carburanti ed un ampio spettro di *bulding block* per l'industria chimica. L'utilizzo di una fonte organica rinnovabile, in alternativa alle risorse fossili, intende altresì creare un circuito virtuoso che valorizza i prodotti di scarto delle produzioni agro/industriali (e.g. industrie alimentari) per il reinserimento nella filiera produttiva dei composti utili all'uomo.

Un vettore energetico innovativo che ha riscontrato particolare attenzione ultimi decenni è il butanolo. Rispetto all'etanolo, un ben noto biocombustibile, il butanolo mostra diversi vantaggi: è meno volatile, ha un potere calorifico inferiore maggiore, è meno igroscopico (può essere facilmente trasportato nelle attuali infrastrutture per comustibili di natura fossile) (Cascone, 2008). Il butanolo trova altresì utilizzo come solvente nell'industria cosmetica, come intermedio chimico nella produzione del butilacrilato, come estraente nell'industria farmaceutica. Il butanolo può essere prodotto per sintesi chimica (petrol-butanolo) o per via fermentativa (bio-butanolo).

La fermentazione Acetone-Butanolo-Etanolo (ABE) è caratteristica di una particolare classe batterica i Clostridia in grado di utilizzare di- e mono-saccaridi -sia esosi che pentosi- comunemente presenti in biomasse lignocellulosiche, in residui delle produzioni agro-alimentari, e in diverse reflui industriali. La specie utilizzata è stata *Clostridium acetobutylicum* DSM 792. *C. acetobutylicum* presenta un particolare *pathway* metabolico. La crescita in condizioni discontinue è caratterizzata da due fasi ben distinte: acidogenesi e solventogenesi. Nella fase iniziale di acidogenesi le cellule si riproducono e producono acidi acetico e acido butirrico. La produzione degli acidi causa comporta una diminuzione del pH nel terreno di coltura e le condizioni ambientali cominciano a diventare sfavorevoli per la crescita. La seconda fase, di solventogenesi, si attiva per pH inferiori a 4.7-4.5 ed è accompagnata da uno shift morfologico e metabolico: le cellule diventono endospore, non sono più in grado di duplicarsi, assimilano gli acidi e producono i solventi Acetone-Butanol-Etanolo in rapporto molare, tipicamente, 3:6:1. La massima concentrazione di butanolo prodotta riportata in letteratura è 13g L^{-1}, oltre esso è tossico per le cellule poiché penetra la membrana cellulare e causa la morte delle cellule. Negli ultimi anni i diversi sviluppi ottenuti nell'ambito della biologia molecolare e nell'industria di processo hanno rinnovato l'interesse per la produzione di butanolo per via fermentativa (Qureshi and Ezeji, 2008). In particolar modo al fine di massimizzare la produttività del processo e renderlo economicamente competitivo diversi studi si stanno recentemente indirizzando verso la produzione di butanolo tramite reattore a letto fisso. Questa particolare configurazione reattoristica vede la co-presenza di cellule acidogeniche e solventogeniche. L'ottimizzazione della configurazione a letto fisso richiede quindi necessariamente la conoscenza delle cinetiche di entrambe le tipologie cellulari.

Scopo della tesi. Lo studio svolto durante il DdR ha riguardato il processo di produzione Acetone-Butanolo-Etanolo (ABE) per via fermentativa a partire da fonti rinnovabili. Il lavoro si è articolato in quattro linee: analisi di mercato di potenziali substrati, pretrattamento della biomassa/substrato, fermentazione e produzione ABE, analisi tecnico-economica del processo produttivo. L'attività si è svolta presso il Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale dell'Università degli Studi di Napoli Federico II e presso la "Enco" Consulting Company, Napoli. Una parte delle attività è stata svolta presso l'Università di "Western Ontario"- Canada.

Analisi di mercato e studio di fattibilità tecnico economica

Lo studio è stato condotto in collaborazione con la "Enco" Consulting. L'analisi di mercato è stata condotta al fine di individuare possibili feedstock utilizzabili per la produzione di butanolo per via fermentativa. Infatti, il costo del substrato del processo di fermentazione ABE rappresenta circa il 60% dei costi totali di produzione. Quindi la ricerca di substrati economici, disponibile a portate in grado di soddisfare le produzioni ABE industriali rappresenta uno dei fattori chiave dell'intero processo. Data la stagionalità di alcuni prodotti/scarti, un altro obiettivo è l'individuazione di un spettro di substrati che garantiscono continuità al processo produttivo ABE. Quattro differenti tipologie di scarto sono state analizzate:

-Siero lattiero caseario;

-HSCB (High Sugar Content Beverage). Bevande commerciali ad alto contenuto zuccherino;

-Biomassa lignocellulosica. Intesa come colture dedicate;

-Residui lignocellulosi delle lavorazioni agricole. Scarti arborei derivanti dalle lavorazioni e dalle raccolte di prodotti agricoli;

-Waste food. Scarti umidi derivanti dal settore alimentare.

La massima produzione di butanolo è stata stimata considerando la disponibilità europea di ogni singolo feedstock, la composizione media, e la rese di butanolo riportate in letteratura. Tale valore è stato poi comparato alla richiesta europea di carburante. L'analisi ha riportato che circa il 20% della richiesta europea di *gasoline* potrebbe essere sostenuta utilizzando scarti derivanti dal settore alimentare e tale valore arriva al 60% se si considerano le biomasse lignocellulosiche. La produzione massima di butanolo riportata è da considerarsi come valore massimo teorico poiché la conversione parziale degli zuccheri e la perdita di solvente in fase di recupero e concentrazione costituiscono inevitabili perdite di produttività.

Lo studio di fattibilità tecnico-economica della produzione di butanolo è stato sviluppato con riferimento a biomassa lignocellulosica. Simulazioni del processo su piattaforma Aspen Plus® sono state integrate a stime economiche condotte in accordo con le metodologie classiche dell'ingegneria di processo. Il processo di produzione del butanolo è stato diviso i tre sezioni: pretrattamento – fermentazione - recupero. Particolare attenzione è stata posta alla fase di simulazione del pretrattamento. Essa ha incluso:

- sminuzzamento della biomassa in un'unità di chipping/milling;
- *steam explosion;*
- idrolisi acida e idrolisi enzimatica in unità reattoristica.

Il costo delle principali unità è stato stimato in accordo alle correlazioi riportate in Peters et al. (2003) e aggiornate al 2011 in accordo all'*index cost*. L'intero processo è stato caratterizzato in termini di potenziale economico EP definito come la differenza tra valore del prodotto e i costi.

Assumendo il costo del butanolo di 0.6 € Kg^{-1} e produzione annuale di 2100 tonn yr^{-1}, il potenziale economico del processo è di 1,250 K€ yr^{-1} assumendo nullo il costo del substrato.

La differenza tra il potenziale economico stimato e il tasso di deprezzamento del investimento principale fissato (420 k€ yr^{-1}), lascia spazio per la fattibilità del processo.

Pretrattamento della biomassa/substrato

L'attività è stata condotta presso l'Università "Western Ontario" con riferimento ad una specifica biomassa lignocellulosica: *corncob*. La biomassa selezionata è il residuo della produzione di mais ed è caratterizzato da un elevato contenuto di glucani e xilani, circa 37% e 27% rispettivamente (Lee et al., 2011). Il pretrattamento è stato condotto in accordo ad un processo innovativo con uso di una nuova classe di solventi: Deep Eutectic Solvent (DES). I DES sono composti in genere da due sali non tossici ed economici che quando miscelati danno luogo a liquidi poiché la forte interazione intramolecolare (e.g. legami idrogeno) conferisce alle miscele solide un punto di fusione inferiore a quello dei singoli sali (Zhang et al., 2012). La maggior parte dei DES sono economici, biodegradabili, non tossici, in grado di solubilizzare composti organici e sono non volatili a temperatura ambiente. I DES sono caratterizzati da proprietà chimico-fisiche simili a quelle dei ben noti liquidi ionici, ma a differenza di quest'ultimi non sono tossici e sono molto più economici. Infatti, i costi dei DES maggiormente investigati sono: ~ 65 $US/kg per cloruro di colina, ~ 20 $US/kg for urea, ~ 35 $US/kg for glicerolo. I costi sono decisamente inferiori a quelli del liquid ionici (e.g. ~ 240 $US/Kg per 95% cloruro de1-butil-3-metilimidazolio). Il pretrattamento investigato ha previsto:

-Trattamento della *corncob* con DES. Sono stati investigati tre DES: cloruro di colina-glicerolo, cloruro di colina-urea, cloruro di colina–imidazolo. I test con ciascun DES sono stati condotti a tre temperature di esercizio: 80°C, 115°C, e 150°C.

-Recupero dei prodotti della biomassa pretrattate mediante antisolventi. Sono stati investigati due antisolventi: acqua ed etanolo.

-Caratterizzazione della biomassa recuperata in termini di concentrazione di inibitori, glucani, xilani, lignina solubile ed insolubile, indice di cristallinità (XRD).

-Idrolisi della biomassa trattata.

I principali risultati ottenuti sono stati:

a) La biomassa trattata con il DES costituito da cloruro di colina-imidazolo a 150°C ha presentato la maggiore riduzione della concentrazione di lignina insolubile.
b) L'utilizzo di etanolo come antisolvente si è dimostrato più efficace in termini di recupero della biomassa rispetto all'acqua.
c) La concentrazione di inibitori (acido acetico, HMF e furfurale) formati durante il processo di pretrattamento è di circa 0.20 g L^{-1}, ben inferiore ai valori critici per la successiva fermentazione (Wood et al., 2014).
d) Il pretrattamento con cloruro di colina-glicerolo a 150°C è caratterizzato da digeribilità della cellulose raggiunge il 91.5% e l'idrolisi enzimatica avviene in 10h raggiungendo 15g L^{-1} di glucosio.

I risultati riportati evidenziano che i DES sono una valida alternativa, più economica e sicura, dei ben noti liquidi ionici, per il pretrattamento di biomasse lignocellulosiche.

Fermentazione e produzione ABE

Presso l'Università di Napoli "Federico II" è stata allestita la produzione di butanolo tramite fermentazione ABE (Acetone-Butanolo-Etanolo). L'attività è stata finalizzata alla caratterizzazione delle cinetiche di acidogenei e solventogenesi, con riferimento alla crescita cellulare e alla produzione di butanolo. Sono state allestite ed esercite due configurazioni reattoristiche.

- Un CSTR a pH controllato è stato allestito al fine di caratterizzare la cinetica delle cellule acidogeniche. Xylosio, uno dei principali zuccheri presenti nella biomassa lignocellulosica, è stato utilizzato come substrato. Il pH è stato fissato a 5.5 e prove sono state condotte al variare della velocità di diluizione (D). Il processo di conversione è stato caratterizzato in termini di conversione dello xylosio, concentrazione cellulare e concentrazione degli acidi.
- Un CSTR equipaggiato con un'unità di microfiltrazione è stato allestito al fine di caratterizzare la cinetica delle cellule in fase di solventogenesi. Due campagnie di prove sono state allestite: una con xylosio come substrato al fine di integrare i dati ottenuti con le cellule acidogeniche cresciute in xylosio; una con lattosio come substrato al fine di integrare i dati ottenuti con le cellule acidogeniche cresciute su lattosio e riportate in un precedente lavoro dello stesso gruppo di ricerca (Napoli et al., 2011). L'unità di microfiltrazione permette di contenere le cellule nonostante la velocità di diluizione sia nettamente superiore alla velocità netta di crescita delle cellule. Al variare della velocità di diluizione il processo di conversione è stato caratterizzato in termini di concentrazione di substrato, solventi e concentrazione cellulare.

I risultati ottenuti sono stati riportati di seguito:

a) In condizioni di acidogenesi le cellule che crescono su xylosio hanno raggiunto una μ_{MAX} = 1.2 h^{-1} e una costante di emivelocità massima K_{XYL} =0.07 g L^{-1}. Il valore di μ_{MAX} ottenuto è confrontabile con quello caratteristico della crescita su lattosio. Il valore della costante K_{XYL} è due ordini di grandezza inferiore rispetto a quello riportato con lattosio (Napoli et al., 2011). Questo conferma quanto riportato in letteratura che *Clostridium* metabolizza zuccheri monomerici più facilmente che il disaccaride lattosio.

b) In condizioni di solventogenisi la massima produttività di butanolo è stata: 0.4 g L h^{-1} a D=0.10 h^{-1} per le prove con lattosio come fonte di carbonio; 1.7 g L h^{-1} a D=0.17h^{-1} per le prove con xylosio come fonte di carbonio.

c) L'elaborazione dei dati di produzione di butanolo unitamente ad un modello pseudo-globale proposto per l'attività cellulare in condizioni di solventogenesi ha permesso di stimare la velocità specifica di produzione di butanolo.

I risultati ottenuti sono in fase di trasferimento per l'implementazione di un modello di simulazione di un reattore a biofilm a letto fisso. La configurazione reattoristica permetterà di aumentare le prestazioni del processo produttivo.

SUMMARY

In the last decades, the global warming, the increase of the crude oil price, and legislative restrictions regarding the use of non-renewable energy sources increased the interest in the biotechnology route for biofuels production. The interest is included in a wider picture: the biorefinery concept of renewable resources. Indeed, the aim is the exploitation of renewable resources to produce fuels, power, heat, and value-added chemicals from biomass conversion. The production of butanol – an advanced biofuel (Cascone, 2008) - according to the fermentation route from waste(water) streams well fit into the biorefinery concept. However, the industrial development of the butanol production by the biotechnological route is still limited by several issues: i) the high cost of the substrate; ii) the end-product inhibition (Zeng et al., 1994); iii) the high product recovery cost (Napoli et al., 2012a; Wua et al., 2015).

The study carried out during the Ph.D. program aimed at investigating the butanol production process by fermentation from renewable resources. The activities were articulated according to three paths: i) feedstock market and techno-economic feasibility assessment of butanol production; ii) biomass pretreatment; iii) butanol production and characterization of the ABE fermentation process. The investigation was carried out at the Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale of the University of Naples 'Federico II' in cooperation with the "Enco" Engineering Consulting Company. The activities regarding the biomass pretreatment was carried out at the "University of Western Ontario" Canada.

Waste to energy: feedstocks market assessment

The cost of the substrate represents about 60% of the overall production cost for a fermentation process. Therefore, feedstock availability at high mass rate, constant availability over the year and low cost is one of the key issue for the success of the butanol production. A survey of potentials "waste biomass" – solid and liquid streams – as feedstock for butanol production was carried out. The maximum butanol production rate from each biomass-feedstock candidate was estimated taking into account the feedstock availability rate, the average composition and the butanol yield.

The market assessment for the feedstock was part of a wider analysis regarding the techno-economic feasibility of butanol production from lignocellulosic biomass. A potential flowsheet to produce butanol by conversion of a lignocellulosic biomass was simulated by means of the software Aspen Plus®. The production process was split into three sections: the upstream section, the fermentation section, and the butanol recovery section. Particular attention was paid to the upstream process. The units of the potential flowsheet were analysed according the approximated cost-estimation methods integrated with the simulation software Aspen Plus®.

Biomass Pretreatment

A new class of solvents DES (Deep Eutectic Solvent) was investigated to obtain fermentable sugars from corncob. Corncob - a byproduct of corn grain production - is currently being used as a potential feedstock for cellulosic ethanol production in the United States because it is charcaterized by low lignin content and high carbohydrate content. The fatures of DESs are very interesting: they exhibit physico-chemical features similar to ionic liquids; they are more environmentally friend and much cheaper then iolic liquids. The DES-pretretated corncob was characterized in term of lignin content, inhibitors concentration, crystallinity index and enzymatic digestibility.

Butanol production and characterization of the ABE fermentation process

The study was aimed at contributing to get a kinetic framework to support the butanol production by *C. acetobutylicum* from simple sugars. The kinetic framework

is a key issue to optimize continuous biofilm reactor that are characterized by the presence of an heterogeneous cell population: the kinetics of acidogenic and solventogenic cells are investigated. Xylose and lactose were investigated as carbon source. Xylose is one of the main components of the lignocellulose hydrolysates; lactose is they main component of the cheese-whey, a wastewater stream released from the cheese industry. The kinetics of acids production by acidogenic cells and butanol production by solventogenic cells were investigated using *ad-hoc* reactor configurations: CSTR under controlled pH and CSTR with microfiltration unit respectively. Operating conditions of the continuous tests were also selected to maximize the butanol production and butanol selectivity.

1. SCIENTIFIC BACKGROUND

1.1 Introduction

The exploitation of renewable energy sources has gained renewed and increased interest in recent years. The interest is fuelled by a issue manifold that include: the global warming; the continue fluctuation of the oil prices; tha fluctuation of the fossil features due to instability of suppliers; the non-perennial availability f fossil fuels. The use of organisms growing on organic substrates and able of producing solvents is pursued by years time. Nevertherless the unquestionable advantages, the butanol production has not yet reached the industrial maturity. Further investigation is reuired to analyze the entire production process, to characterize main bottlenecks and to optimize the overall production process. Main issues to be investigated are:

- Alternative substrates for fermentation
- Upstream processing
- The use of mutant strains
- Reactor design and optimization to support industrial scale-up
- Downstream processing

1.1.1 Biorefinery Concept

In the last decade, innovations and new tools - e.g. genetic engineering, Analysis of Metabolic Fluxes, Transcriptomics and Proteomics, Life Cycle Assesment - have expanded the knowledge of biobutanol production process and have offered improvement for the overall production process. In particular, the attention/focus has been moved from the production/optimization of a specific item to the production/optimization of a process/plant aimed at the exploitement of all fractions of feedstocks: the kernel of the biorefinery concept.
The biorefinery concept is analogous to petroleum refinery, which produce fuels and basic products for the petrochemical industry as alkenes and polycyclic aromatic hydrocarbons. The main goal of the biorefinery is the exploitement of all component of a feedstock by producing a spectrum of of items - fuels, power, heat, and value-added chemicals – and reducing – or better, avoiding - the production of waste(water) streams. From the industrial point of view, a biorefinery is a pool of plant/processes aimed at the combined production of the listed items. The principal sections of a biorefinery are:

- Upstream processing units (pretreatment of biomass)
- (bio)conversion units to produce fuels, power, heat, and value-added chemicals production (fermentation process units)
- Downstream processing units (to recovery and concentrate products)

The biorefinery creates a positive circuit that allows waste agricultural, industrial and food to be reintegrated into the production chain of compounds useful to man.

1.2 Feedstocks

1.2.1 Substrates Assessment

The ability of saccharolytic clostridia to utilize a wide spectrum of carbohydrates have stimulated research in the use of alternative cheaper feedstocks (Gapes, 2000; Ezeji et al., 2004a). Wastewater streams, e.g. the agro-industrial wastewater streams, are characterized by high carbohydrate content and they require expensive treatments before to be disposed. Therefore, the use of these wastewaters as substrate can be a valid alternative to conventional-expensive feedstocks because they are for free or, better, their processing may be associated with income for the remediation.

Cheese-whey. The industrial processing of milk for the production of butter and cheese releases considerable stream of wastewater. Indeed, 4-5 kg of waste are produced for each kg of cheese. The wastewater coming from the manufacture of cheese is typically known as cheese-whey. The organic fraction of this stream is remarkable high: the Biochemical Oxygen Demand (BOD) is about 2000 mg L^{-1}, and the COD is about 50-70 g L^{-1} (Najafpour et al., 2008). The Italian law allows the discharge in the sewer system wastewater characterized by COD lower than 500 mg L^{-1} (D.Lgs.152/06). Wastewaters characterized by higher values must be treated to remove pollutants. For discharge in surface waters (rivers, etc..) the permissible limit is 160 mg L^{-1} (D.Lgs.152/06). A small dairy produces 20m^3 day^{-1} of wastewaters. These numbers increase significantly if we consider that in Italy there are about 2,800 dairies, producing 8 to 10 million m^3 yr^{-1} of waste (clal, 2013). The disposal problem, which is cost-effective, can be overcome according to different procedures. One chance is the use of cheese-whey as a dietary supplement for farm animals. The concentrated cheese-whey mixed with cereal is an excellent ingredient for the formulation of complex fodder. However, the quantity of cheese-whey is too large and the overaal amount can not be disposed according to this path. A second chance is the fractionation: the lactose may be used in the chemical and pharmaceutical industries, the proteins-whey - concentrated and dried – may be used in the food industry, and in particular in the confectionery industry. The main issues related to the use of cheese-whey as a feestock for the food industry are storage and transport. Indeed, the high sugar content of the cheese-whey causes spontaneous fermentation. Therefore, the transformation plants must be located as close as possible to the dairy. The cheese-whey is also an optimal substrate for fermentation. The main advantages of cheese-whey as substrate for fermentation process are: the good spatial and time availability, the possibility to find suppliers in a small confined region. These characteristics positively affect the economic aspect of the biobutanol production process.

High sugar content beverages (HSCBs) – e.g. fruit juices, syrups, soft drinks, and sport drinks - are a potential carbon source for the ABE fermentation. They contain sugars as sucrose, fructose, and glucose. A huge quantity of them are yearly disposed. The disposal of industrial wastewater streams and expiry date beverages is a critical issue for the high carbon content. As reported by Dwidar et al. (2012) bioethanol is successfully produced adopted carbonated beverages as carbon source. The preliminary tests to produce butanol from a carbonated beverage were very promising and the characterization of butanol production by adopting high sugar content beverages is recently reported by Raganati et al., 2015.

Lignocellulosic material

They are the most abundant renewable resource on the earth (Lopez-Contreras, 2003). One of the positive aspects about the use of lignocellulosic biomass as a substrate is based on the logic of "carbon neutral". Fossil fuels are derived from processes that have taken place over several thousand years. Burning fossil fuels makes a net addition of CO_2 in the atmosphere because the ecosystem can not assimilate the CO_2 produced with the same rate with which it is released. This leads to an increase of the CO_2 in the atmosphere and then to global warming. For this reason, fossil fuels are defined "carbon positive". The biomass is defined "carbon (almost) neutral" because when used as fuel it releases the CO_2 assimilated to grow. However, it is necessary to take into account the CO_2 produced by ancillary processes: harvesting, transport, processing, and distribution for biofuels produced from biomass.

Main components of lignocellulosic biomass are lignin, cellulose and hemicellulose. Cellulose is a polysaccharide consisting of a linear chain of several hundred to many thousands of D-glucose units. It exists in different forms characterized by a spectrum of degrees of polymerisation and molecular weight. Hemicelluloses are made up of shorter heteropoly saccharide chains that consist of mixed pentosans and hexosans The main components are D-xylose, L-arabinose, D-glucose, D-glucuronic, D-mannose and D-galactose. Flickinger and Drew (1999) reported that about 20-40% of lignocellulosic biomass is hemicellulose, with D-xylose being the major component.

C. acetobutylicum is capable of metabolizing all the prevalent sugars present in wood hemicellulose and cellulose hydrolysates (Ezeji et al., 2007a).

Wood is the most abundant natural sources . The wood can be classified in hard wood and soft wood. Table 1 reports the main differences between hard woods and soft woods. A quite large issues spectrum should be taken into account for the selection of the wood as feedstock for the biobutanol production. Main issues are: spatial/time availability, the growth time of the tree species, the demand for the species for other purposes and uses, transport costs, chemical-physical characteristics in relation to fermentation aim.

Italian forest area is about 10.637.589 hectares: about 34.7% of the national territory. In Italy there are 12 billion trees (about 1.500 m^2 of forest for every Italian). The most common tree in Italy is the beech with more than one billion pieces especially in the Apennines. The beech growth sepends on rainfall, humidity, exposure to sun, etc.. It grows about 1 cm yr^{-1} (WWF, 2013). Although beech is very present in Italy, it grows slowly and hence it isn't very available. Instead, the poplar is a good compromise between presence in the Italian regions and its fast growth. The poplar grows between 25-35cm yr^{-1} compared to the average of 9 $cmyr^{-1}$ of the Italian forests. Poplar cultivation in Italy takes place between 9 and 12 years; after dejection of the trees the land can be used for planting other poplars or used for other crops. In the last census in 2000 in Italy 120,000 hectares of poplar groves were present (www.ISTAT.it). More than 70% of the poplar groves in Italy are located in the Po Valley. The total amount of poplar wood is around 45-50% of the national production of timber. 250 tons of wood are obtained from 1 hectare subjected to a ten-year cycle. We can get from a mature poplar: 500 Kg of veneer (for the processing of plywood), 100 Kg of sub-shearing (generally used for the pallet), and 300 Kg of wood chips, to be allocated to biomass for energy production (Panguenta, 2014).

	Hard wood	Soft wood
Definition	Comes from deciduous trees that drop their leaves every year.	Conifer trees have needles, normally do not lose them.
Uses	Used for trimmings and furniture but less frequently than softwood.	Widely used as woodware for building (homes/cabins) and furniture.
Examples	Mahogany, teak, walnut, oak, ash, elm, aspen, poplar, birch, maple etc.	Pine, spruce, cedar, fir, larch, douglas-fir etc.
Cost	Hardwood is typically more expensive than softwood.	
Growth	Slower	Fast
Properties	Broad leaves; enclosed nuts; higher density: not all hardwood is hard e.g. poplar and basswood.	Less dense; less durable; high calorific values;coniferous trees.
Type	Mostly deciduous. Some European evergreen trees that yield hardwoodare holly, boxwood and holm oak.	Evergreen.
Density	High	Low
Shedding of leaves	Hardwoods shed their leaves over a period of time	Softwoods tend to keep their leaves throughout the year.
Found in regions	Trees supplying hardwood are found throughout the world from the Boreal and Taiga forests of the North to the tropics and down into the far South.	Found in the northern hemisphere.
Colour	Dark	Light
Anular ring	Not Distinct	Distinct
Weight	Heavy	Light
Strenght	Strong in compression, tension and shear (strong along and across the grains)	Strong in tension but weak in shear (strong along the grains)
Structure	Non - resinous and close grained	Resinous and splits easy
Fire Resistance	high	Poor
Conversion	Difficult	Easy

Table 1. Comparison between Hard wood and Soft wood (FAOSTAT)

A class of substrate belonging to the “short rotation forestry” is the Miscanthus. It is a grass. It grows very well on poor lands up to 3m in height. It is a perennial plant, therefore it does not need to be replanted every year. The Environmental Research Institute of Wales estimates that if the Miscanthus was planted on 10% of European cultivable land it could provide up to 9% of the electricity consumed by the continent. Currently Miscanthus crops are carried out by ENEA in Sicily (www.enea.it).
The wood is very abundant in the Italian territory but the main disadvantage as substrate for fermentation processes is the cost related to the transport, the harvesting and the pretreatment (Huijgen et al., 2010).

Agricultural residues are waste materials produced from the processing and crop of grain, corn, and flowers, but also grass and straw. Their features are:

seasonal production, high dispersion on the ground, high humidity. Seasonal production makes the availability of the material not constant over the year. The dispersion in the territory increases the transport costs. The humidity degree is also a technical issue. Winter crops are collected in the summer and their humidity degree is low because they are natural dried by the sun. The summer crops are characterized by an humidity degree of about 20-30%. Cereal straw is the main by-product derived from crop, it is collected after the reaping in June and July. The straws are packed in cylindrical bales (apparent density of 100 -120 Kg/m^3) or prismatic bales with a high density (120 -150 Kg/m^3)(ENEA2009). The class of waste material from agricultural residues includes arboreal residues, e.g. pruning of olive groves, vineyards and orchards. On one hand, the availability of the agriculture residues is low. On the other hand, the cost of its pretreatment is low too (Guragain et al., 2011) and this is a key economic issue.

1.2.2 Pretreatment

Pretreatment step of lignocellulosic materials is mandatory in the biomass-to-butanol conversion production. The objective of the pretreatment is to render cellulose and hemicellulose accessible to both chemical and enzymatic hydrolysis for efficient bioconversions. Main goals of the pretreatment are:
i) remove the lignin;
ii) increase the surface area and porosity to facilitate the penetration of hydrolysis agents;
iii) obtain the depolymerisation of hemicellulose.
Several processes have been investigated for the treatment of lignocellulosic biomass (corncob, corn stover, switchgrass, poplar…). As reported in the literature, the agricultural residues are easier to be treated than hardwood materials.
Several technologies are available for the pretreatment of biomass and they strongly affect performances of the successive enzymatic hydrolysis and microbial conversions. The pretreatment processes must be: efficient, chep and environmental friendly. Moreover, some treatments can result in the formation of microbial inhibitors: furfural, hydroxymethyl furfural (HMF), and acetic, ferulic, glucuronic, ρ-coumaric acids (Ezeji et al., 2007b) that are harmful to the ABE fermentation. For this reason attention must be paied at the inhibitor concentration at the end of the pretreatment step. Fig. 1 shows the pretreatment steps mandatory for a fermentation process from lignocellulosic biomass.

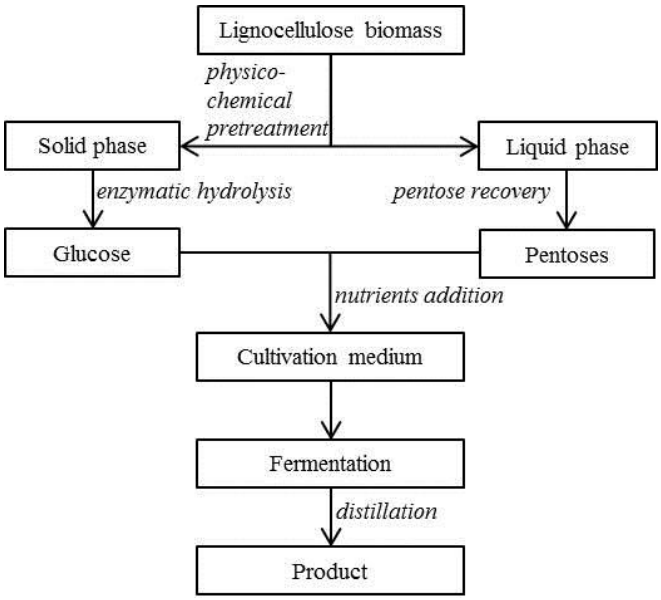

Figure 1. Pretreatment steps for a lignocellulosic biomass to produce butanol

Main lignocellulosic pretreatments are reported in the following.

Strong acid hydrolysis

Concentrated strong acids such as H_2SO_4 and HCl are powerful agents for cellulose hydrolysis (Sun and Cheng, 2002). Moreover, no enzymes are required after the acid hydrolysis. High monomeric sugar yields are obtained at mild temperature conditions. However, disadvantages of concentrated acid pretreatments are: the corrosive nature of the process environment and the acid recycle to reduce costs.

Alkaline hydrolysis

The major effect of alkaline pretreatment is the removal of lignin from the biomass. According to Sun and Cheng (2002), the alkaline hydrolysis mechanism is based on saponification of intermolecular ester bonds crosslinking xylan hemicelluloses and other components such as lignin. Usually, calcium hydroxide and sodium hydroxide ammonia are used. The mild conditions produces a high lignin solubility, especially for biomass with a low lignin content such as softwood and grasses. Furthermore, degradation of sugars to furfural, HMF and organic acids is limited.

Organosolv

Organosolv processes use an organic solvent or mixtures of organic solvents with water for lignin removal. These solvents include ethanol, methanol, acetone, and ethylene glycol. The temperature of the process can be as high as 200°C, but lower temperatures may be sufficient depending on the type of biomass (Ghose et al., 1983). A critical issue about this pretreatment is that the solvent itself can be an inhibitor for the enzymatic hydrolysis and fermentation steps. For this reason, the solvent must be removed prior to fermentation. Moreover, removal and recovery of the solvent is required for reducing process cost and environmental impact.

Biological pretreatment

Several microorganisms such as white, brown and soft rot-fungi are investigated to degrade hemicellulose and lignin. Advantages of biological pretreatments are mild operation conditions and low energy requirement. However, the rate of biological hydrolysis is lower than chemical hydrolysis, so this pretreatment requires long residence times (Cardona and Sanchez, 2007).

Steam explosion

Steam explosion is one of the most applied pretreatment processes. High-pressure saturated steam is injected into a reactor filled with biomass. During the steam injection, the temperature rises to 160-260ºC. Pressure is suddenly reduced and the biomass has an explosive decompression. Under these conditions, hemicellulose degradation and lignin matrix disruption is obtained.

Steam explosion has one critical issue: the formation of degradation products that may inhibit fermenation processes (Garcia-Aparicio et al., 2006).

Room Temperature Ionic Liquids (RTILs)

Ionic liquid are liquid salts at temperature as low as room temperature. There are different RTIL: they are made of an inorganic anion and an organic cation. The difference in the molecular structure renders the bonding of the ions weak enough for the salt to be liquid at room temperature (Rantwijk, 2003). The dispersion of lignocellulosic biomass in RTILs produces the separation of lignin and the increase of cellulose accessibility under ambient conditions and with no use of acid or alkaline solutions. Despite the high potential, main disadvantages of this method appears is the high toxicity and costs of ILs.

1.3 ABE fermentation Process

1.3.1 History

The production of solvents by fermentation dates back to the First World War. At that time the demand of acetone was very high since it was used for the production of cordite, an explosive in which acetone was an important component. Acetone- Butanol-Ethanol (ABE) fermentation process was developed in 1912 at Manchester University by the Russian chemist C. Weizmann. He installed the first production plant for the production of acetone from starch and he used a particular class of bacteria isolating a particular species: *Clostridium acetobutylicum* (Durre, 1998). At the end of the war, since there was not more demand for acetone, the production process was abandoned. After a short time, however, the focus shifted to the butanol and its ester, butyl acetate. Indeed, the automobile sector recorded an increase and these solvents were used as lacquers for finishing the bodywork of cars (Durre, 1998). New butanol production plants were built between 1924 and 1927, in 1945 66% of the total butanol and 10% of the total acetone production were obtained by ABE fermentation, making it the largest scale bioindustry ever run, second only to ethanol fermentation (Jones and Woods, 1986). Later, in the 1960 the production of acetone and butanol by fermentation was abandoned due to the advent of the petrochemical industry. The main issues regarding the ABE process are: the low yields, sluggish fermentations and problems caused by product inhibition. Nowadays, most n-butanol is produced chemically from petroleum sources by the oxo process from propylene (with H_2 and CO over a rhodium catalyst), or by adol process from acetaldehyde (Brekke, 2007).

1.3.2 Solventogenic Clostridia

The genus Clostridium is a heterogeneous collection of gram-positive, spore-forming, obligatory anaerobic, rod-shaped bacteria. The Clostridia can grow on different substrates because they have a wide spectrum of enzymes which break polymeric carbohydrates into monomers that are assimilated in the metabolism. These enzymes include α-amylase. α-glycosidase, glucoamylase, pullulanase, and

amylopullulanase (Ezeji et al., 2007a). Solventogenic Clostridia are able to produce interesting industrial solvents (Acetone Butanol Ethanol) according to the fermentative process known as ABE. Under batch conditions, the fermentation of Clostridia goes according to two successive separate phases: the acidogenic phase and the solventogenic phase (Fig. 2). During the acidogenic phase cell grow and produce acids and gas: butyrate (butyric acid) and acetate (acetic acid), carbon dioxide and hydrogen. The production of the acids causes a decrease in the pH, therefore environmental conditions are no more favorable to bacterial growth. *Clostridium acetobutylicum* adapts to these new conditions by a metabolic and morphological shift: i) the exponential growth phase ends; ii) the active cells become endospores, unable to grow; iii) the acids are converted to solvents, Acetone-Butanol-Ethanol (in a typical molar ratio 3:6:1); iv) the substrate is also converted directly in solvents, Acetone-Butanol-Ethanol. The fermentation process ends because high concentration of the solvents inhibits the process: cell membranes are solubilized and cell death. Therefore, there is a limitation to the maximum solvent concentration – about 2 wt% - that can be achieved during fermentation (Durre, 1998).

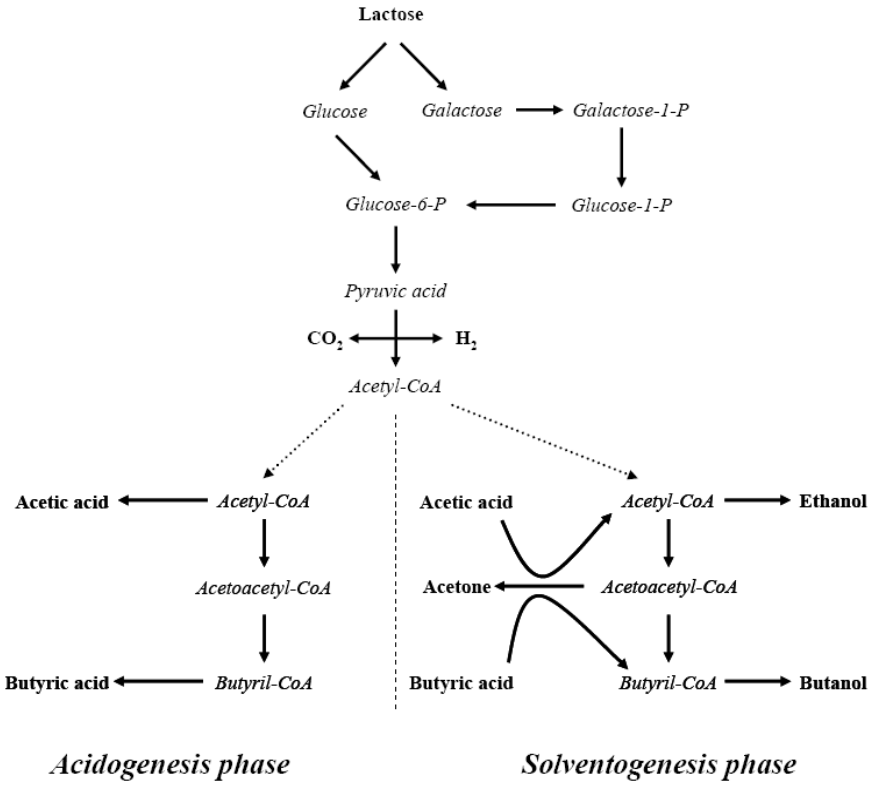

Figure 2. Simplified metabolism of solventogenic Clostridia

As regards the regulation of the butanol fermentation pathways, the control of electron flow in the glycolytic pathway plays a fundamental role. The ferrodoxin oxidoreductase is an enzyme present in clostrdia and employed in the oxidation of products such as NADH FADH produced during the solventogenic phase. Alteration in the direction of electron flow around reduced ferrodoxin can produce a change in the type and quantity of fermentation products (Ezeji et al., 2007b). Researchers have tested the effect of numerous reducing compounds as carbon dioxide (gassing) and methyl viologen. Butanol and ethanol formation were increased at the expense

of acetone synthesis in the presence of these electron carriers (Mitchell, 1998; Zverlov et al., 2006; Gapes, 2000). There are four principal groups of solventogenic Clostridia: *Clostridium acetobutylicum* (fig.3), *C. beijerinckii, C. saccharobutylicum, and C. saccharoperbutylacetonicum.*

C. acetobutylicum grows on starch-based substrates. It is the best-studied solventogenic Clostridium and many improved strains have been developed from this specie (Durre, 1998). There are a number of patented strains that are able to produce solvent at concentrations up to 14-18 g L^{-1} with solvent yields of 25-30% (Shaheen et al., 2000). *C. beijerinckii, C. saccharobutylicum and C. saccharoperbutylacetonicum* are known as the saccharolytic strains and they are phylogenetically interconnected.

The majority of the saccharolytic industrial strains belong to *C. beijerinckii species. C. beijerinckii* grows and produces solvents at a wider pH range. It can utilize a wider variety of carbohydrates as substrate due to its genetic potential. Qureshi et al., (2000) suggested that *C. beijerinckii* is less susceptible to acid crash and therefore more suitable for longer (continuous) fermentations than *C. acetobutylicum. C. beijerinckii* BA101, a mutant strain created by mutagenesis of *C. beijerinckii* NCIMB 8052, gives total ABE concentrations of 14.8-26.1g L^{-1} with yields of 37-50% (Lee and Blaschek, 2001).

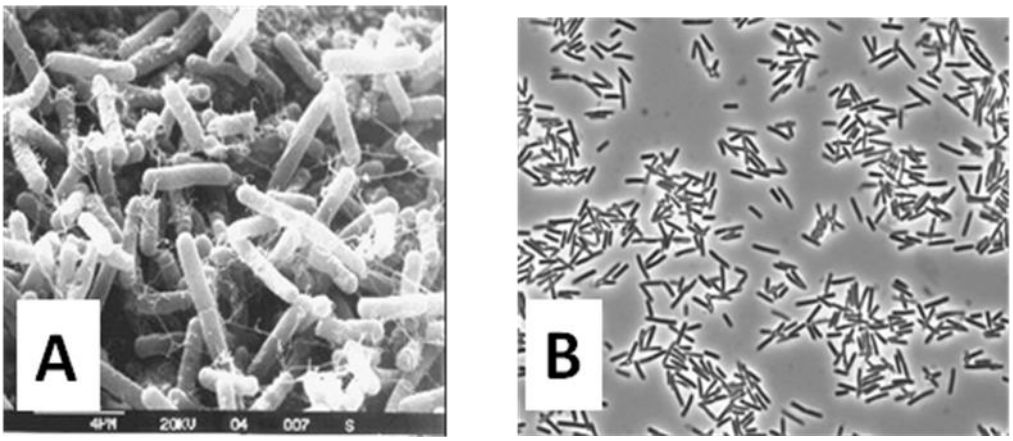

Figure 3. *Clostridium acetobutylicum* observation at A) scanning electronic microscope B) optical microscope (kindly obtained by Prof.ssa Rachele Isticato)

C. saccharobutylicum and C. saccharoperbutylacetonicum are the less studied strains. Shaheen et al., (2000) studied these strains in a comparative fermentation and they found that the fermentation performance is better on glucose and molasses than on maize. The average solvent concentration was 19.6g L^{-1} with a yield of 30%.

1.4 Butanol Production

1.4.1 Overview of butanol

Butanol is a four carbon alcohol (C_4H_9OH) that have four structural isomers. The isomer of greater commercial interest is n-butanol. Butanol can be produced via chemical synthesis (petro-butanol) and by fermentation (bio-butanol). The butanol can be used as a fuel (Cascone, 2008) but also, it can be used as a solvent (for paints, coatings, varnishes, resins, gums, dyes, camphor, vegetable oils, fats, waxes, shellac, rubbers and alkaloids), coating (as a solvent for a variety of applications,

such as lacquers), plasticizer, textile (as a swelling agent and manufacturing garments from coated fabric). Moreover it can be used in cosmetics (including nail care products ,eye makeup, lipsticks, shaving products and personal hygiene products), hydraulic fluids, drugs, antibiotics, hormones, and vitamins, as a chemical intermediate in the production of butyl acrylate and methacrylate, and as an extractant in the manufacture of pharmaceuticals (Dow, 2006).

1.4.2 Butanol as biofuel

Butanol is an excellent biofuel thanks to its various chemical and physical properties. Some features may be related to those of the ethanol of gasoline. Butanol has 4 carbon atoms, then +25% energy with respect to ethanol and it is 6 time less evaporative than ethanol and 13.5 time less evaporative than gasoline. Butanol is less hygroscopic than ethanol and it can be transported in existing transport systems of gasolines (Lee, 2008a). Butanol can be mixed at high ratios with gasoline for use in existing cars without modifications in gasoline-designed engines. Indeed, Environmental Energy Inc., a U.S. company owner of a patent for biobutanol production, claims that butanol can be used as a total replacement for gasoline without any modifications to car engines (Brekke, 2007). Butanol is characterized by higher viscosity and lower octane number than ethanol. Tab.2 reports properties of common (bio)fuels and butanol

Characteristic	Gasolin	Butanol	Ethanol	Methanol
Formula	C_4-	C_4H_9O	CH_3CH_2O	CH_3O
Boiling Point (°C)	32-210	118	78	65
Energy Density (MJ kg^{-1})	44.5	33.1	26.9	19.6
Air Fuel Ratio	14.6	11.2	9.0	6.5
Research Octane Number	91-99	96	129	136
Motor Octane Number	81-89	78	102	104
Heat of Vaporisation (MJ	0.36	0.43	0.92	1.20

Table 2. Properties of common (bio)fuels and butanol

1.4.3 Butanol production in the world

The production of butanol by fermentation at large scale is still a real challenge. Although the process optimization asks for a deep characterization, a few research groups and companies are currently involved in the process investigation. Main issues to be addressed are:

i) the high cost of the substrate;
ii) the low product concentration and productivity in fermentation due to end-product inhibition (16-18 g L^{-1} due to solvent toxicity);
iii) the high product recovery cost.

Highlighted issues can be overcome by application of modern molecular techniques, genetic manipulation to the solventogenic Clostridia, computational engineering, downstream processing and process integration. Indeed, the simultaneous optimization of the various points of the entire process can lead to a significant increase in biobutanol concentration, yield and recovery.

As regards companies two large companies - BP and DuPont – joined in a large enterprise aimed at producing butanol. They claimed that their technology will be competitive as long as the crude oil price remains above $80 per barrel (Scott and Bryner, 2006). Figure 4 reports the worldwide distribution of plants producing butanol.

Three units are producing butanol via thermochemical route. The biotechnological route appears more challenging. It has also known that some plants should be built-up in Brazil in the near future. In 2005, David Ramey drove a Buick fuelled by pure butanol across the United States. Emissions of CO, hydrocarbons and NOx were reduced substantially compared to gasoline though the consumption increased by 9%. Environmental Energy Inc. (EEI), Ramey's company, is planning to produce Butyl Fuel™ via a newly developed fermentation process involving two Clostridia species (Ramey and Yang, 2004). In 2006, BP and DuPont announced a joint venture to bring to market the next generation in biofuels. The first product will be biobutanol, which was targeted for introduction in 2007 in the United Kingdom (UK) as a gasoline bio-component (DuPont, 2006). They claim that their technology will be competitive as long as the crude oil price remains above 80 $ per barrel (Scott and Bryner, 2006).

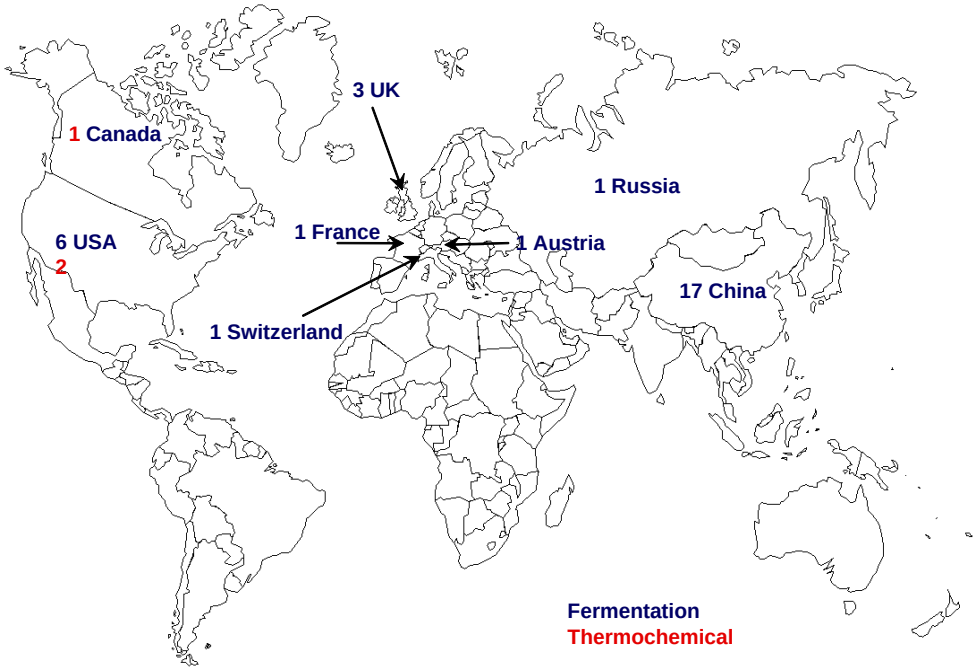

Figure 4. Map of industrial and pilot-plants producing butanol.

In cooperation with British Sugar, an existing ethanol plant in the UK will be converted into a biotechnological butanol production facility, and a feasibility study is already under way to examine the possibility of constructing larger facilities in the UK (DuPont, 2006). The production of biobutanol from specifical lignocellulosic biomass seems promising and it is on the agenda for a number of companies (Antoni et al., 2007). The Europe is still late with respect to the USA and China.

In Italy no plant has been built-up so far. Therefore further studies should be made to make the sector more attractive for companies willing to address capital. In 2007 a research program aimed at the design/optimization of the butanol production by fermentation process started at the University of Napoli. The activity carried out until 2010 is reported in the PhD thesis of Fabio Napoli (2010). The study aimed at the assessment of both the kinetics of cell growth and metabolites produced during the lactose fermentation by *C. acetobutylicum*. The lactose was used as carbon sources to emulate the cheese whey, an abundant lactose-rich wastewater. Continuous cultures were carried out under a wide interval of operating conditions in order to characterize the fermentation process under acidogenesis (Napoli et al., 2011) and the solventogenesis phases (Napoli et al., 2009). Moreover, Napoli

developed a first version of continuous biofilm reactor for the ABE production (Napoli et al., 2010). The activity carried out between 2011 and 2014 was carried out in order to obtain butanol from renewable resources as lignocellulosic biomass (dedicated colture, agriculture residues) and waste water streams (HSCB, cheese-whey). An exhaustive overview of this activity is reported in the PhD thesis of Francesca Raganati (2014). The activities were articulated according three paths: i) the characterization under batch conditions of the ABE fermentation process as regards kinetics and yields using monomeric sugars (glucose, xylose, arabinose, mannose, saccharose, fructose) to emulate lignocellulosic biomass; ii) the characterization under batch conditions of the ABE fermentation process as regards kinetics and yields using high sugar content beverages as carbon source; iii) the butanol continuous production by biofilm fixed bed reactor using cheese whey as carbon source.

1.4.4 Reactor Configuration

In the last twenty years different reactor configurations have been investigated to enhance the productivity of ABE fermentation process. Indeed, design of bio-reactors play an key role in the biotechnological industry. The knowledge of the rate of reactions and operating conditions is necessary to improve reactor performances and productivities (Maddox, 1989; Qureshi and Blaschek, 2001). Several batch reactors have been adopted in early stage of ABE fermentation studies. Large scale batch fermentation was used during the 1940s and 1950s. Usually, the biobutanol production on industrial scale was carried out in large batch fermentors ranging in capacity between 200 and 800 m^3. The main disadvantages of this reactor typology are:

a) low productivity;
b) the long downtime characteristic of discontinuous fermentation;
c) lost of substrate converted in cells.

Of course, the ABE fermentation process can not be economically competitive if carried out according to traditional conditions. As reported in literature (Tashiro et al., 2004; Ezeji et al., 2004b) different studies regarded the fed batch technique. Unfortunately, the results were not promising as regards the improvement in the productivity and solvent yield.

The long downtime characteristic of the discontinuous fermentation may be overcome adopting continuous culture. This configuration may be characterized by solvents concentration and yield as large as batch cultures. Furthermore, they may be characterized by improved productivity. The main disadvantage of this operation modality is related to the process stability. One of the first continuous butanol production process was carried out by Leung and Wang (1981). They operated a free cell reactor with *C. acetobutylicum* ATCC 824 and using glucose-limited (50 g L^{-1}) as carbon source. They operated at dilution rate of 0.1 h^{-1} and 0.22 h^{-1}. At D 0.1 h^{-1} they obtained yield of 0.32 g g^{-1} and a productivity of 1.5 g $L^{-1}h^{-1}$. At dilution rate of 0.22 h^{-1}, a maximum productivity of 2.55 g $L^{-1}h^{-1}$ was obtained, but the solvent yields reduced to 0.26 g g^{-1}.

Two continuous fermentation systems have been investigated by Mutschlechner et al. (2000) in order to separate the growth phase (acidogenesis) from the production stage (solventogenesis). The cell growth was maximized in the first fermentor, acids production occurred in the first fermentor, and solvent production occurred in the second fermentor. *C. beijerinckii* NRRL B592 was used and glucose was adopted as carbon source. The first fermentor was operated at a

dilution rate of 0.12 h^{-1} and the second was operated at 2.2 10-2h^{-1}. Solvent concentrations of ~15 g L^{-1} (4.8, 9.1, and 0.9 g L^{-1}, acetone, butanol, and ethanol) were obtained. Solvent yield and productivity were 0.25 g g^{-1} and 0.27 respectively. The final solvent concentration was close to that obtained in batch fermentations. On the basis of the promising results, a pilot-plant has been developed in Austria (Nimcevic and Gapes, 2000). However, studies regarding continuous free cells cultures are characterized by low specific productivity. Because of the low growth rate of cells under solventogenesis conditions, very low dilution rate must to be selected. For this reason, the process intensification by bioreactor design is required to improve the whole process. Process intensification can be achieved by:

i) confainement of the biocatalyst within the reactor, by means of semipermeable membranes;
ii) immobilizing the microbial cells on a support.

Membrane reactors are characterized by the confinement of cells into the reactor. Under these conditions, cells concentration may be theoretically increased up to any values. The cell concentration in the reactor is the result of a delicate equilibrium between growth rate under solventogenic conditions and cell died rate. Recently, Zheng et al., 2013 have reported a continuos fermentation with MF unit to butanol production. They use *C. saccharoperbutylacetonicum* N1-4 and xylose as strain and carbon source, respectively. Interesting results were obtained: maximum butanol productivity (3.32 g L^{-1} h^{-1}) was observed at a dilution rate of 0.78 h^{-1}, approximately 6-fold higher than observed in continuous culture without cell recycling (0.529 g L^{-1} h^{-1}). However, the spread of this reactor at industrial scale level is still limited by the cost of the membrane that suffers of biofouling. Recently some studies focused on butanol production by means of biofilm reactors. This reactor configuration is a technology adopted since the beginning of the last century in several biotechnological processes (Kolot, 1984) and mainly investigated as regards wastewater bioremediation. Results available in literature on the butanol productivities in biofilm reactors are very promising. Qureshi and Blaschek (2000) have reported solvents productivity of 15.8 g $L^{-1}h^{-1}$ when glucose is adopted as carbon source. Napoli et al. (2010) produced up to 4.5 g $L^{-1}h^{-1}$ in a biofilm packed bed reactor fed with a lactose based medium. Raganati et al. (2013) have reported butanol productivity 2.66 g $L^{-1}h^{-1}$, when cheese-whey was used as carbon source. Immobilized cell systems have several advantages:

i) the physical retention of the cells in the reactor;
ii) the high cell concentrations, allowing greater productivity.

The main disadvantage of this reactor typology is related to gas formation and the transport phenomena within the biofilm. Indeed, gas (hydrogen and carbon dioxide) formed during the anaerobic fermentation may accumulates in the biofilm as microbubbles and may detach biofilm pieces. Gas accumulation into the bioparticles may induce their floating and segregation with respect to the medium flow. Furthermore, the biofilm is characterized by two different regions: inner and external region. The first one is characterized by a substrate concentration lower with respect to the external region values. Similarly, the products concentration in the inner region is larger with respect to the bulk values. Because of simultaneous presence in the biofilm of two different types of cells, the knowledge of the kinetics – cell growth rate, butanol production, etc. - of both of them is necessary to design and optimize the whole bioconversion process.

2 AIM OF THE THESIS

The study carried out during the present Ph.D. program aimed at investigating the butanol production process by fermentation from renewable resources. The activities were articulated according to three paths:

i) feedstock market and techno-economic feasibility assessment of butanol production;
ii) biomass pretreatment;
iii) butanol production and characterization of the ABE fermentation process.

The investigation was carried out at the Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale of the University of Naples 'Federico II' in cooperation with the "Enco" Engineering Consulting Company. The activities regarding the biomass pretreatment was carried out at the "University of Western Ontario" Canada.

Waste to energy: feedstocks market assessment

The cost of the substrate represents about 60% of the overall production cost for a fermentation process. Therefore, feedstock availability at high mass rate, constant availability over the year and low cost is one of the key issue for the success of the butanol production. A survey of potentials "waste biomass" – solid and liquid streams – as feedstock for butanol production was carried out. The maximum butanol production rate from each biomass-feedstock candidate was estimated taking into account the feedstock availability rate, the average composition and the butanol yield.

The market assessment for the feedstock was part of a wider analysis regarding the techno-economic feasibility of butanol production from lignocellulosic biomass. A potential flowsheet to produce butanol by conversion of a lignocellulosic biomass was simulated by means of the software Aspen Plus®. The production process was split into three sections: the upstream section, the fermentation section, and the butanol recovery section. Particular attention was paid to the upstream process. The units of the potential flowsheet were analysed according the approximated cost-estimation methods integrated with the simulation software Aspen Plus®.

Biomass Pretreatment

A new class of solvents DES (Deep Eutectic Solvent) was investigated to obtain fermentable sugars from corncob. Corncob - a byproduct of corn grain production - is currently being used as a potential feedstock for cellulosic ethanol production in the United States because it is charcaterized by low lignin content and high carbohydrate content. The fatures of DESs are very interesting: they exhibit physico-chemical features similar to ionic liquids; they are more environmentally friend and much cheaper then iolic liquids. The DES-pretretated corncob was characterized in term of lignin content, inhibitors concentration, crystallinity index and enzymatic digestibility.

Butanol production and characterization of the ABE fermentation process

The study was aimed at contributing to get a kinetic framework to support the butanol production by *C. acetobutylicum* from simple sugars. The kinetic framework is a key issue to optimize continuous biofilm reactor that are characterized by the presence of an heterogeneous cell population: the kinetics of acidogenic and solventogenic cells are investigated. Xylose and lactose were investigated as carbon

source. Xylose is one of the main components of the lignocellulose hydrolysates; lactose is they main component of the cheese-whey, a wastewater stream released from the cheese industry. The kinetics of acids production by acidogenic cells and butanol production by solventogenic cells were investigated using *ad-hoc* reactor configurations: CSTR under controlled pH and CSTR with microfiltration unit respectively. Operating conditions of the continuous tests were also selected to maximize the butanol production and butanol selectivity.

3 WASTE TO ENERGY

This section is focused on techno-economic issues.
The first part regrads the the review of the feedstock for the butanol fermentation. The focus was on feedstocks charcaterized by availability at high mass rate, constant availability over the year, and no competition with the food market.
The second part of the section was focused on the assessment of the techno-economic feasibility of butanol production from lignocellulosic biomass by means of the software Aspen Plus® integrated with approximated cost-estimation methods.

3.1 Renewable Feedstocks for Biobutanol Production by Fermentation

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Present contribution proposes a survey of biomass feedstocks for butanol production having the European fuel market as reference. Several feedstock typologies have been taken into account. The maximum butanol production rate from each feedstock has been estimated by processing the feedstock availability rate, the average composition – raw material and pre-treated material - and the butanol yield. The study regarded: waste(water) streams reach of sugars and lignocellulosic biomass.

Introduction

The rising global energy demand, increased greenhouse gas emission, continued consumption of fossil fuels and uncertainties related to oil price, have led to an increasing interest in the production of renewable energy sources such as biofuels. Butanol – an alternative fuel - has gained a renewed attention in recent years. Butanol -with respect to ethanol- has higher energy content, lower volatility and it is less corrosive (Lee et al., 2008a; Jin et al., 2011). In this scenario, the production of n-butanol by Clostridia strains, commonly known as ABE (acetone–butanol–ethanol) fermentation is one of the most widespread industrial fermentation processes (Papoutsakis 2008; Zheng et al., 2013; Procentese et al., 2015). Acetone-Butanol-Ethanol are produced during the last stage of the batch fermentation of different Clostridium strains (*Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium aurantibutyricum*). Batch fermentations are characterized by two phases, acidogenic phase and solventogenic phase. During the acidogenic phase the cells grow and produce acids (butyrate and acetate). The production of acids causes a decrease in pH and the environmental conditions become unfavourable to bacterial growth. The active cells become endospores, unable to grow and the acids are converted to solvents: Acetone–Butanol–Ethanol in a typical molar ratio 3:6:1 (Jones and Woods, 1986). The cost of the substrate represents about 60% of the overall production cost for a fermentation process. For this reason, feedstock available at high mass rate, with a constant availability over the year and low cost is a key issue

for the success of the butanol production (Gu et al., 2014). Different Clostridia strains are able to metabolize pentoses and hexoses sugars and several study are reported in literature about the pretreatment of biomass to obtain fermentable sugars (Banerjee et al., 2011; Zhang et al., 2011; Gao et al., 2014). Instead, to the authors knowledge, only few studies are reported in literature regarding the effective amount of feedstock available to biofuel production (Melikoglu et al., 2013; Noor et al., 2013; Kiran et al., 2014). Furthermore, in these few studies just one kind of waste (e.g. food waste, municipal waste…) is taken in account.
This contribution reports a survey of several potential feedstocks available for butanol production by biotechnological route. Moreover, the butanol yield have been taken into account to assess the potential butanol production rate. The total butanol productivity has been compared to the European fuel demand.

European fuel market

A market analysis has been conducted with reference to the demand of fuel in Europe. Table 1 reports the fuel (diesel and gasoline) consumption in the European Union (ENERDATA, 2014). Assuming that the fuel demand is constant in the future and that 20% of the fuel in the 2020 must be from renewable resources (European Directive 2009/28/CE), in Europe it will be necessary to have a biofuel stock of about 40 Mt yr^{-1}: 2/3 diesel-like and 1/3 gasoline-like.

Fuel	**2008**	**2009**	**2010**	**2011**
Diesel	199	196	199	200
Gasoline	98	95	90	86

Table1. Fuel consumption rate (Mt yr^{-1}) (ENERDATA, 2014)

Feedstock market assessment

Five potential feedstocks, available for butanol production by biotechnological route were taken into account: cheese-whey, high sugar content beverage, lignocellulosic biomass, agriculture residues and waste food.

Cheese-whey

The industrial process of milk for the production of butter and cheese produces considerable wastewater streams. The processing of 1 Kg of milk produces 0.2 Kg of cheese and 0.8 kg of wastewater, known as cheese-whey. The organic fraction present in the wastewater is remarkably high: the Biochemical Oxygen Demand (BOD) is about 2 g L^{-1}, and the Chemical Oxygen Demand (COD) is about 50-70g L^{-1} (Najafpour et al., 2008). In particular, the COD is larger than the limit set by the European commission – 250-500 mg L^{-1} - for discharge in sewer systems (75/440/EEC). Therefore, the cheese-whey must be treated to reduce the COD. To understand the impact of the cheese-whey as pollutant it is sufficient to know that a small dairy produces about 20 m^3 day^{-1} of wastewaters, a pollution comparable to a community of about 10,000 people. A total of 40×10^3 Kt yr^{-1} of whey is produced in the European Union (Molella et al., 2013). The disposal of the cheese-whey is cost-effective and it may be reduced by adopting the cheese whey as a feedstock for industrial processes. In particular, the cheese-whey may be adopted as: i) a dietary supplement for farm animals; ii) a source of lactose to be adopted in confectionery industries. The latter fate is very interesting from the economic point of view but the market is not particularly large and the investment for the production is quiet high. It

is clear that a large fraction of cheese-whey must be disposed with other processes like the fermentation to produce butanol (Napoli et al., 2011; Raganati et al., 2013).
The main advantages of cheese-whey are: the continuous production over the year at almost constant rate and the production in narrow regional area. Both features positively affect the economic aspect of the process to produce butanol. The main issues related to the use of cheese-whey are storage and transport. Indeed, the high sugar content causes spontaneous fermentation. Therefore, the plant must be located as close as possible to the dairy.

High sugar content beverages

High sugar content beverages (HSCBs) - such as fruit juices, syrups, soft drinks, and sport drinks - may be a potential carbon source for ABE fermentation. Dwidar et al. (2012) and Raganati et al. (2015) have proved that the HSCBs are a good candidate to produce butanol. Indeed, these beverages contain a wide spectrum of sugar (e.g. sucrose, fructose, etc.). The market size in Europe of the HSBC is of the order of $70 \bullet 10^6$ m^3 yr^{-1} and it is possible to assume that about 0.1% becomes a stream to be remediated (about $1 \bullet 10^6$ $m^3 yr^{-1}$).
The main advantages of HSCBs are: the continuous production over the year at almost constant rate. The main issue related to the use of HSCBs is the transport cost, indeed, their production isn't localize in a focused area.

Dedicated lignocellulosic biomass

Lignocellulosic biomass is one of the most abundant natural sources. The main advantage of the use of lignocellulosic biomass as a fermentation substrate is the presumed "carbon quasi-neutral" feature. Indeed, the biomass-derived fuels are defined "carbon quasi-neutral" because their combustion releases about the CO_2 fixed during the biomass growth. The CO_2 required for the fuel production (biomass harvesting, pre-treatment processes, fermentation processes, biofuel recovery and concentration) makes the carbone balance positive (Farrell et al., 2006). Main issues to take into acout for the selection of the lignocellulosic biomass as feedstock for the butanol production are: the spectrum of cultivar available in each country, the growth time of the cultivars, the demand for each cultivar for other purposes, harvesting cost, transport and pre-treatment costs (Huijgen et al., 2010). Depending on the selected species, the next step is the selection of the pre-treatment process.
The lignocellulosic species present in Europe and at the attention for biofuel production are the short rotation plantations. Short rotation plantations can be classified in two different specialized forestry systems: short rotation forestry (SRF) and short rotation coppice (SRC). Both of them consist of high-yielding varieties and tolerating several rotations. The differences between the two systems are: the period of rotation - from 8 to 20 years for SRF and from 2 to 4 years for SRC - and the species. The species included in SRF in central Europe and Scandinavian countries are: poplar, aspen, alder, and birch. The species included in SRC are: willow and poplar. Willow is mainly produced in Sweden, Finland, Denmark, the Netherlands, UK, and Ireland. Poplar and robinia are cultivated in warmer climates such as Mediterranean area (Italy, France and Spain) (Uslu et al., 2010).
The EU Forest Action Plan promotes the use of forest materials as an energy source. However, one of the main pillars of forestry policy is the principle that the forest should not decline. Many European countries have specific legal measures that support these objectives. For instance, law 43/2003 of woodlands in Spain defines principles and goals for forest management. The Swedish Forest Industries

Federation Climate Manifesto indicates the increasing extraction of biofuel from the forest from the current 7 TWh to 20 TWh annually.
Figure 1 shows the total roundwood production in the EU-28 between 2005 to 2012 (EUROSTAT 2014). The roundwood production is almost constant at 315 Mt yr^{-1}, and approximately the half of the rate is from Germany, France, Finland and Sweden. The 21% of total amount was wood fuel to produce energy and the rest was used by the forest based industries to produce wood-realted products (sawnwood, wood panels, pulp and paper). According to Uslu et al. (2010), the forest industry in the European Union will approximately use 400Mt $year^{-1}$ of roundwood in 2020.
The main advantage of lignocellulosic biomass is the availability rate. However, this exploitement of this renewable resource is damaged by the transport costs.

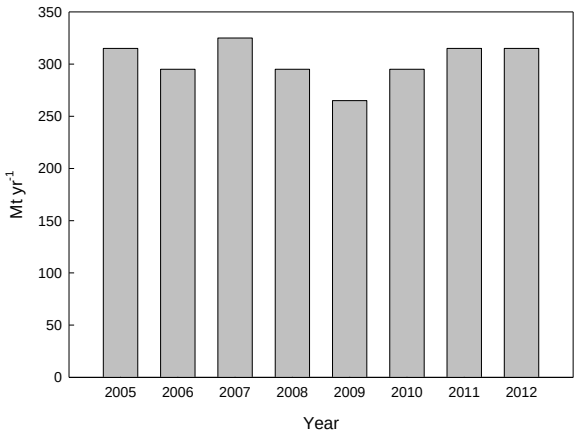

Figure 1. Annual European roundwood production (EUROSTAT, 2014)

Agricultural residues

Agricultural residue-wastes are produced during lignocellulosic harvesting processes and during crops. Lignocellulosic harvesting processes include arboreal residues, e.g. pruning of olive groves, vineyards and orchards. Crop-derived wastes include residues of grains, corns, flowers, grass and straws. Main relevant features of this typology of residues are: seasonal production and high humidity. Seasonal production makes the availability of the material not constant over the year. The availability rate of agricultural residues is approximately 150 Mt yr^{-1} (Bairati, 2012). The cost of the agricultural residue pretreatment is lower than that of the lignocellulosic dedicated biomass.
The availability of agricultural residues substrate is low when compared with the lignocellulosic dedicated biomass.

Food loss and food waste

The annual global food waste is estimated to be about 1.3 billion tons, about one third of the total food production intended for human consumption (FAO, 2011). The waste problem along “the food chain” was presented several years ago to the European Parliament. On October 2010, the “Declaration Against Food Waste” was presented in Brussels. The declaration of the Parliament and the European Commission was to promote strategies and resolutions aimed at reducing food waste by at least 50% by 2025. The report “Strategies for a More Efficient Food Chain in the EU” was drawn up and approved in 2012.
Recently, the Swedish Institute for Food and Biotechnology (SIK, 2012) proposed a distinction between food loss and food waste. Food loss “takes place during

agricultural production, post-harvest, and processing stages in the food supply chain". The lost is due to climatic and environmental factors and accidental causes that can be traced back to the limitations of agricultural technology and infrastructures used in some geographic areas. Food waste occurs "at the end of the food chain (distribution, sale and final consumption): "the food waste comes from behavioral factors and intentional choices, based on which perfectly edible food is discarded and thrown away". Moreover, both food loss and waste refer to products intended for human consumption, excluding animal feed and non-edible parts of plants. A comparison of food lost and waste in European and non European country pro capita is reported in Tab. 2. The highest value is in United Kingdom and United States (about 110 Kg yr^{-1} per capita), the lowest in Sweden (72 Kg yr^{-1} per capita).

Nation	Food loss and waste (Kg per capita)
United Kingdom	110
United States	109
Italy	108
Germany	82
France	99
Sweden	72

Table 2. Food loss and waste: comparison between European and no European states (Buchner et al., 2012)

The quantity of annually wasted food in Europe is about 89million tons. Fig.2 shows the different distribution of waste food (Buchner et al., 2012). Household fraction (42%) of the food-waste is the main contribute to the food waste. The fraction of food waste from the food processing (39%) and the catering and restaurant services (14%) are also remarkably (Buchner et al., 2012). The food waste is: i) safe, ii) available, iii) cheap. The reported features of the food waste pointed out that the food waste may be taken into account as substrate candidate for ABE fermentation to butanol production.

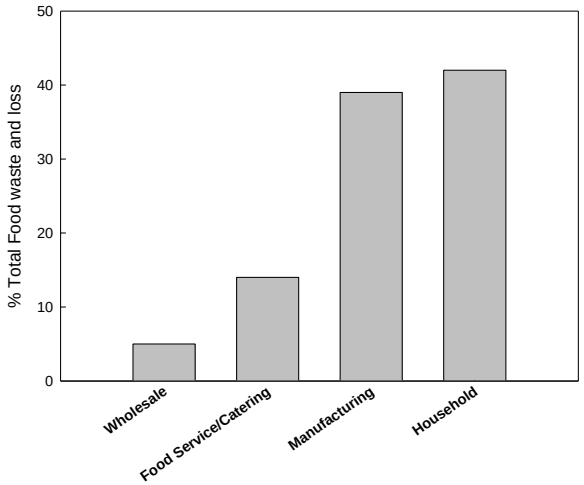

Figure 2. Food loss and waste distribution(Buchner et al., 2012)

Substrate expected cost

The disposal cost of an organic compound depends on COD content. Table 3 reports the substrate expected cost for the disposal of cheese whey and HSCB assessed according the following relationship (Eco Center, 2014):

$$\text{Substrate Expected Cost [€/ton]} = 9 + 0.0005*\text{COD}[\text{mg/kg}_{\text{waste}}] \quad (1)$$

The COD values were assumed about at 100g L^{-1} and 1000 g L^{-1} for cheese-whey and HSCB respectively.

Butanol expected productivity

The butanol productivity from renewable resources has been assessed taking into account the year availability ($W_{resource}$), sugar composition of the resource with respect to the fermentable sugars, and the butanol yield for each fermentable sugar (Y_B/sugar,i). The maximum butanol production rate ($W_{BUTANOL}$)from each resource may be assessed according to the relationship (2):

$$W_{BUTANOL} = W_{RESOURCE} * \Sigma_{ferm\ sugar} (\omega_{sugars} * Y_{B/sugars}) \quad (2)$$

where $\omega_{sugar,i}$ is the mass fraction of the sugar “i" in the resource.
Main assumptions for the assessment of the maximum butanol production adopting the investigated feedstocks are reported hereinafter.
The lignocellulosic biomass is made of cellulose, hemicellulose and inert. The mass fraction of cellulose and hemicellulose reported by Kumar et al. (2009) was adopted. The main sugar component was adopted for no-lignocellulosic resource (e.g. lactose for cheese-whey).

Lignocellulosic treatment converts all the cellulose into glucose. Lignocellulosic treatment converts all the hemicellulose into glucose, mannose, arabinose and xylose. The mass ratio among the components has been set at 5:1:2:4, respectively. The butanol expected productivity for lingnocellulosic biomass and agricultural residues has been assessed taking into account the fraction of cellulose and hemicelluloses of the biomass.

The butanol yield was set according to data reported in the literature. It was: i) 0.26 $g_{butanol}/g_{sugar}$ for lactose (Napoli et al., 2011); ii) 0.24, 0.15, 0.16, and 0.19 $g_{butanol}/g_{sugar}$ for glucose, arabinose, mannose, and xylose, respectively (Raganati et al., 2014b); iii) 0.16 $g_{butanol}/g_{sugar}$ for HSCB, as an average value assessed from data reported by Raganati et al. (2015); iv) 0.20 $g_{butanol}/g_{sugar}$ for hemicellulose, as an average value taking into account the composition and the butanol yield of each component (Raganati et al., 2014); v) 0.20 $g_{butanol}/g_{sugar}$ for food waste, as an average value taking into account glucose, mannose, arabinose, xylose, lactose, sucrose and fructose yields.

The availability rate of the feedstocks was from several sources (European Commission 2010, ISTAT 2014, ENEA 2014; ENERDATA 2014).
The production rate of cheese-whey was assessed at $40 \bullet 10^3$ Kt $year^{-1}$.
The HSCB market is of the order of $70 \bullet 10^3$ Kt yr^{-1} and 0.1% was taken into account as stream to be disposed/remediated (about 70 Kt yr^{-1}).
The 21% of total amount of lignocellulosic biomass was taken into account as available lignocellulosic biomass rate $63 \bullet 10^3$ Kt yr^{-1}.

The agricultural residue and food waste availability rate have been assessed assuming that the whole European amount of agriculture residues and waste food are used to butanol production.
Table 4 reports an approximate estimation of the maximum butanol productivity assessed with reference to the investigated feedstocks available.

SUBSTRATE	AVAILABILITY RATE	SUGAR COMPOSITION	MAX expected butanol productivity	SUBSTRATE expected cost
Cheese-whey	$40 \cdot 10^3$ Kt yr^{-1}	4% lactose	416 Kt yr^{-1}	- 68 € ton^{-1} (Eq.1)
HSCB	$70 \cdot 10^3$ Kt yr^{-1}	50% (mixture of glucose, fructose, saccarose, ..)	6 Kt yr^{-1}	- 500 € ton^{-1} (Eq.1)
Lignocellulosic dedicated biomass	$315 \cdot 10^3$ Kt yr^{-1}	45% cellulose 30% hemicellulose	$10 \cdot 10^3$ Kt yr^{-1}	n.a.
Agriculture residues	$150 \cdot 10^3$ Kt yr^{-1}	30% cellulose 50% hemicellulose	$26 \cdot 10^3$ Kt yr^{-1}	0
Food waste	$89 \cdot 10^3$ Kt yr^{-1}	50% (mixture of glucose, fructose, saccarose, ..)	$8 \cdot 10^3$ Kt yr^{-1}	n.a.

Table 3. Max expected butanol productivity to be produced in Europe adopting the reported resources.

The total butanol productivity was assessed about at $46 \bullet 10^3$ Kt $yr^{-1}$and compared to the European fuel demand. If industrial streams/food wastes are consider about 10% of the European demand for the gasoline may be replaced by biobutanol. The fraction may rise up to 50% if the lignocellulosic biomass and agriculture residues are adopted. The assessed production rate should be considered as maximum because the total feedstock may not be available, the sugar conversion may not be complete and a fraction of solvent may be lost during the recovery and concentration process.

Conclusion

Several biomass feedstocks for butanol production were investigated. The maximum butanol production rate from each feedstock has been estimated taking into account data of the average composition and butanol yield available in the scientific literature. About 50 % of the European demand for fuel may be replaced by biobutanol. The assessed production rate should be considered as maximum because the sugar conversion may not be complete and a fraction of solvent may be lost during the recovery and concentration process.

Acknowledgment

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3.2 Process Simulation of Biobutanol Production from Lignocellulosic Biomass

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The simulation of a potential flowsheet to produce butanol by conversion of a lignocellulosic biomass is reported in the present contribution. The flowsheet consisted of upstream, fermentation, and downstream sections and it was simulated by means of the software Aspen Plus®. The attention has been focused on the upstream section. The proposed process is characterized by an economic potential three times the depreciation rate of the main fixed investment rate.

Introduction

The production of chemicals and fuels from renewable resources is among targets of the white biotechnology. The interest in the biotechnology route for these productions is due to several driving forces: the global warming, the increase of the crude oil price, and legislative restrictions regarding the use of non-renewable energy sources (Naik et al., 2010). A potential solution to the growing demand for fuels from renewable resources is the butanol produced according to the biotechnological route. Main issues that require further investigation are: i) the scouting of renewable resources to be bioconverted into butanol; ii) the selection of strains characterized by high solvent productivity; iii) the development bioreactor systems characterized by high specific productivity; iv) the development of downstream processing strategies for enhanced solvent recovery.

As regards renewable resources, the aim is to select feedstocks plentiful, inexpensive, and not edible (Qureshi and Blaschek, 2000; Kumar et al., 2009; Raganati et al., 2013). Lignocellulose is the most plentiful renewable resource on the planet, it is made of potential fermentable sugars, it is not useful as food resources, and it is quite cheap. Lignocellulosic materials are characterized by three components: cellulose, hemicelluloses, and lignin. An interesting classification of lignocellulosic feedstocks and their average compositions has been reported by Garrote et al. (1999).

As regards pre-treatment methods, Conde-Mejía et al. (2011) reported a spectrum of single steps typically adopted/suggested for lignocellulosic feedstocks: steam explosion, ammonia fiber explosion, irradiation, dilute acid hydrolysis, and organosolvent extraction.

Regarding to fermentation step, Clostridium strains are able to metabolize a wide range of carbohydrates - like glucose and lactose, pentoses and hexoses - and to produce butanol mixed with acetone and ethanol: the fermentation known as ABE, Acetone-Butanol-Ethanol (Jones and Woods, 1986). Studies carried out on ABE fermentation adopting feedstocks derived from lignocellulosic biomass – a potential inexpensive feedstocks - have reported the yield values and the solvent production rate for batch tests (Raganati et al.,2012; Jurges et al., 2012). It is also known that the fermentation productivity may be strongly increased by process intensification as in biofilm packed bed reactors (Lee et al., 2008b; Napoli et al., 2010; Raganati et al., 2013). Studies available in the literature on solvent recovery are quite limited.

Particularly interesting is the contribution from Liu et al. (2004) regarding downstream process synthesis for biochemical production of ABE. They have proposed a list of optimal and near-optimal flowsheets with conventional operating units. Attempts to characterize the butanol recovery from the experimental point of view (Pinto Mariano et al., 2012; Rom et al., 2013) and from the techno-economic point of view (Napoli et al., 2012a) are also reported in the literature. To the author knowledge, just few studies are reported in the literature on the whole upstream process (Quintero et al., 2011; Qiao et al., 2013). The characterization of the process to produce butanol from the selected feedstock as well as the techno-economic assessment of the process are key issues to be developed in order to identify critical points to study in depth (Olivieri et al., 2013).
The present contribution reports results of a study aiming at investigating the techno-economic feasibility of butanol production from lignocellulosic biomass. The production process has been splitted into three sections: the upstream section, the fermentation section, and the butanol recovery section. Particular attention has been paid to the basic steps required for the upstream process. The upstream units have been analysed according the approximated cost-estimation methods integrated with the simulation software Aspen Plus®.

Materials and Process description

Biomass composition depends on the lignocellulosic culture adopted. An exhaustive review on the biomass composition has been reported by Kumar et al. (2009). A first classification of lignocellulosic cultures is hardwood and softwood. Within each class the concentration of hemicellulose, cellulose and lignin range over a quite limited interval. The average composition of the biomass adopted in the present study is: lignin 20%, cellulose 44%, and hemicellulose 36%.
Figure 1 shows a synoptic diagram with the main steps adopted for the investigated process. They are: the comminution of the biomass; the steam explosion with the release of cellulose, hemicellulose, and lignin; hydrolysis of the cellulose and hemicellulose, hexoses and pentoses as products; lignin harvesting; sugars fermentation; and butanol recovery.

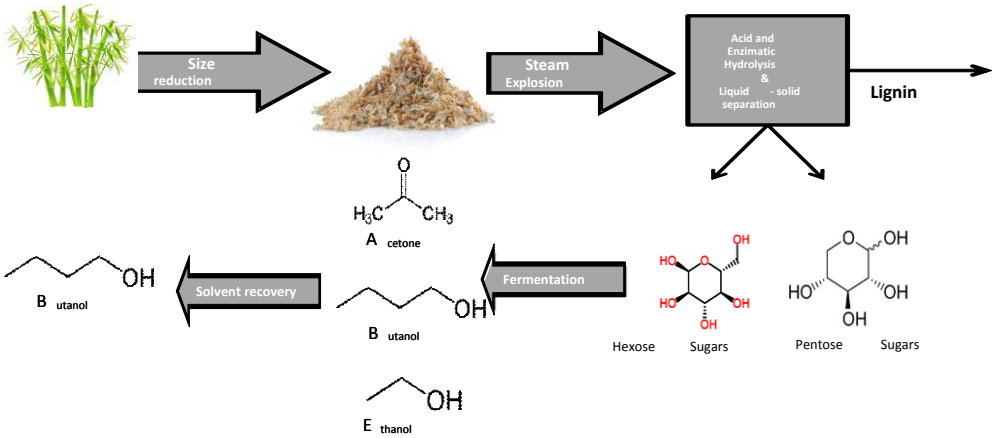

Figure 1. Synoptic diagram of the butanol production form lignocellulosic biomass. Main steps of the upstream process.

The units adopted to produce butanol from lignocellulosic biomass are reported in Figure 2.

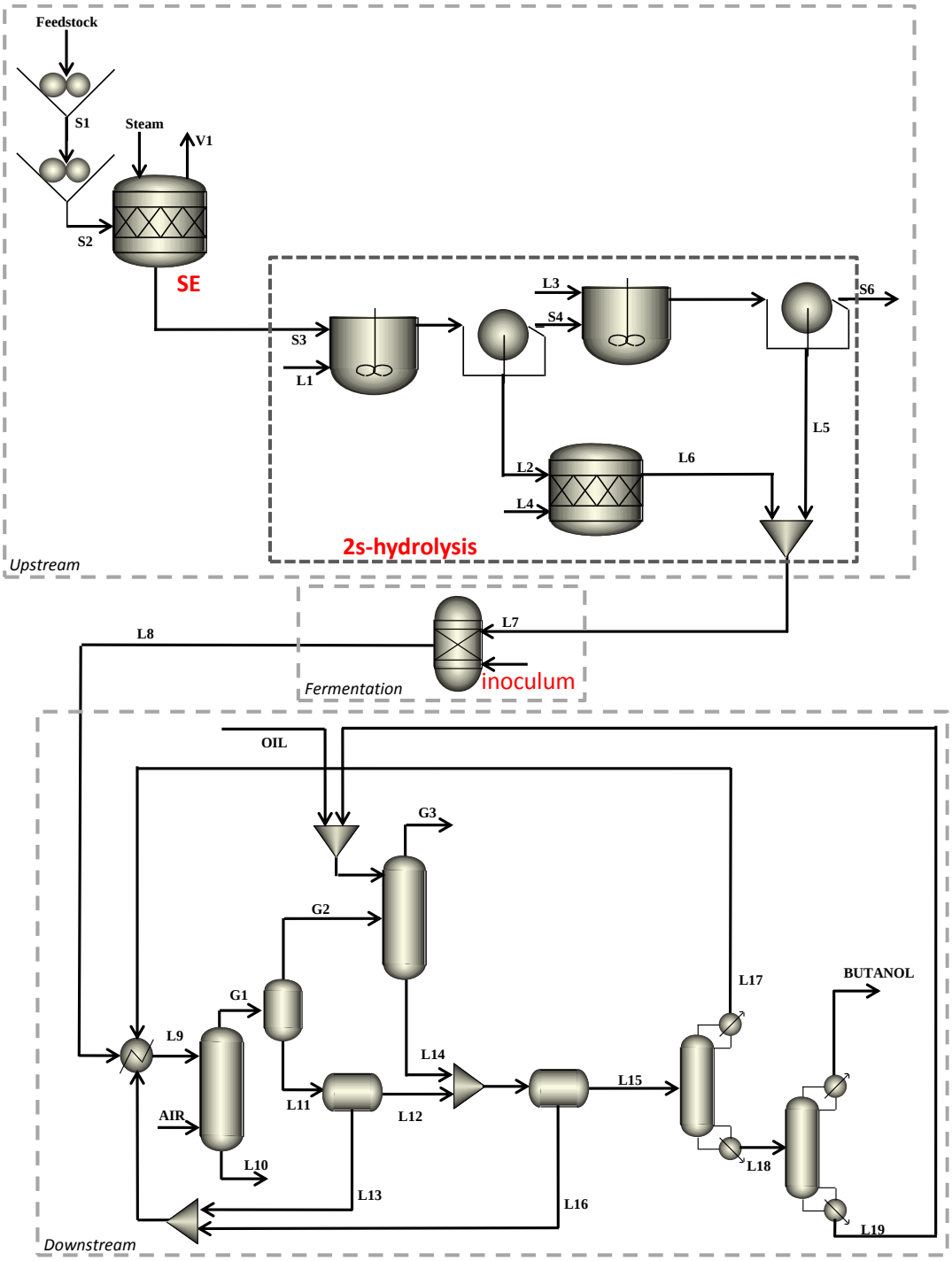

Figure 2. Flowsheet of the investigated butanol process from lignocellulosic biomass. SE) Steam explosion; 2s-hydrolysis) two-step hydrolysis section; Downstream) details in Napoli et al. (2012).

Biomass comminution has been simulated by a train of chipping/milling units able to reduce the size of the softwood from the field-harvesting size to those (S2 stream) optimal for successive operations. The steam explosion operation (SE) has been adopted to pre-treat the comminuted biomass. A saturated steam stream (Steam in Figure 2) at 160°C has been adopted. The steam is vented as stream V1. The suspension of exploded biomass (S3 stream) is sent to a two-step hydrolysis process. The HCl stream L2 is mixed with the suspension S3 to provide the first acid hydrolysis. The hydrolyzed sugars are recovered mainly into the liquid stream L3 and

the suspension rich in cellulose and lignin S4 is mixed with the enzyme rich stream L4 to provide the second enzymatic hydrolysis. The suspension S6 rich in lignin is separated from the liquid stream L5 rich in hydrolyzed sugars. The liquid stream L3 rich in a mixture of pentoses and hesosse produced from the hydrolysis of the hemicellulose is neutralized: the stream L3 is mixed with the stream L4, an aqueous solution of NaOH at 0.4 M. The streams rich in pentoses L6 and that rich in hexoses L5 are mixed to be sent at the fermentation section.
Main assumptions regarding the composition of the solution produced from the upstream section are: i) L6 stream is a solution of glucose, mannose, arabinose, and xylose; ii) products from the partial conversion of the lignin (e.g. phenolic compounds, vanillin, ferulic acid) and from hemicelluloses and cellulose (e.g. furfural and HMF) are absent.
The fermentation unit consists of a biofilm packed bed reactor (Napoli et al., 2010). *Clostridium acetobutylicum* has been selected as the actor of the ABE fermentation because it is able to convert hexoses and pentoses typically released during the hydrolysis of cellulose and hemicellulose (Raganati et al., 2012).
Details on the recovery and concentration of butanol from the fermentation broth are reported in Napoli et al. (2012a).
The cheeping and the milling units have been designed assuming Hardgrove Grindability index value 77 according to Green and Perry (2008).
The filtration unit used to harvest solids has been designed assuming a solid concentration of about 2 Kg/m^3. The area of a vacuum continuous filter has been estimated assuming the pressure drop across the filter 70 kPa and the specific cake resistance $2 \cdot 10^6$ m kg^{-1}.
Reactors adopted for the acid and the enzymatic hydrolysis have been designed adopting the kinetics reported in Liu et al. (2012). The volume of the reactors has been assessed.
The volume of the biofilm reactor has been assessed by setting: i) the sugar conversion at 90%; ii) butanol and ABE yields assessed by Raganati et al. (2012); iii) specific butanol productivity was set at 4.5 g L^{-1} h^{-1} (Napoli et al., 2010).
The flowsheet in Figure 2 has been simulated and sized by means of Aspen Plus®.

Economic assessment

The cost estimation for the conceptual design of the flowsheet dedicated to the production of butanol from lignocellulosic biomass has been carried out following the procedure proposed by Happel and Jordan (1995). The purchased cost for the main units of the flowsheet has been estimated in agreement with correlations reported in Peters et al. (2003). Data have been updated to 2011 by means of the index cost (Peters et al., 2003).
Economic data have been integrated with those assessed for the train of units adopted for the recovery and concentration of butanol from the fermentation broth assessed by Napoli et al. (2012). The overall process has been characterized in terms of economical potential (EP):

$$EP = \text{Product value} - \text{Raw Matl. Cost} \quad (1)$$

and of the yearly rate (L) of depreciation of the main fixed investment (I) required for the plant. L has been estimated as:

$$L = eI_F \quad (2)$$

where e is [1 yr^{-1}] the yearly fractional depreciation rate, I_F the fixed investment of the system (Peters et al., 2003). The depreciation rate “e” has been assessed accordingly to the sinking fund method: $e=i/[\exp(in)^{-1}]$, where n [yr] is the expected

project life and i [1 yr^{-1}] the rate of return of the firm. Tentative reference values of n=10, i=0.10 1 yr^{-1} and e= 0.058 1 yr^{-1} have been adopted for this preliminary economic assessment (Rudd and Watson, 1968).

Results

The structure of the flowsheet reported in Figure 2 allows to assess the investments for the units by focusing on the three subsections separately. According to this strategy the attention has been focused on the first two sub-sections: upstream and fermentation. Data from the assessments carried out by Napoli et al. (2012) have been adopted for the third sub-section (downstream). The flowsheet has been simulated for a flow rate of butanol stream rate fed at the downstream section of set at 251 Kg h^{-1}, according to the simulation carried out by Napoli et al. (2012). Table 1 reports the results of the simulation of the milling/chipping process in terms of the size of "particles". The size range of the biomass adopted (feedstock) has been set according to typical data available in the literature on softwood harvesting. Results particle distribution after the chipping step (S1 stream) and the milling step (S2 stream) are reported.

Size [mm]	Feedstock	S1 stream	S2 stream
0-1	0%	0.7%	20%
1-1.6	0%	11%	80%
1.6-50	0%	9%	0%
50-100	0%	3%	0%
100-150	0%	76%	0%
150-2000	100%	0,3%	0%

Table 1. Particle size distribution of the feedstock and after simulation of both the chipping (S1) and milling (S2) steps.

The power required is 0.02 kWatt and 0.29 kWatt for chipping and milling steps, respectively.The operation units dedicated to the hydrolysis have been sized in terms of volume. The reactor for the acid hydrolysis was 0.57 m^3, that for the enzymatic hydrolysis was 8.8 m^3. The purchased cost for the main items adopted in both upstream and fermentation sub-sections of the flowsheet reported in Figure 2 are listed in the Table 2. Main values of the factors suggested by Happel and Jordan (1995) have been adopted for the items K through I. The economical potential EP of the process for the proposed flowsheet is about 1,254 k€ yr^{-1} assuming that cost of the raw materials is negligible: it is the butanol cost (0.6 € kg^{-1}) for the yearly production rate (2,100 t yr^{-1}).
The depreciation rate of the main fixed investment (L) is about 420 k€ yr^{-1}.

The comparison between EP and L suggests that there is room for successive economic assessment. Additional data would be required to complete the analysis with exercise costs. An optimization of the overall process is required too.

Final remarks

A potential lignocellulosic flowsheet to produce butanol by conversion of a lignocellulosic biomass has been reported. It has been simulated by means of the simulation software Aspen Plus®. Set the butanol production rate at 251 kg h^{-1}, the comparison between the economical potential (1,254 k€ yr^{-1}) and the depreciation

rate of the main fixed investment (420 k€ yr^{-1}) left room for the economic feasibility of the process.

	Item			**Material (k€)**
A	Crusher 1 (chipping)			7.9
B	Crusher 2 (milling)			7.9
C	Steam Explosion unit			20.3
D	Hemicellulose hydrolysis unit			25.7
E	Filter 1 and Filter 2			116.6
F	Cellulose hydrolysis unit			60.0
G	Neutralization Unit			18.9
I	Fermentation			310.0
J	(sum of A to I)			638.2
		factor (*)		
K	Insulation	18.8%	of J	120.0
L	Piping	90.0%	of J	574.0
M	Foundation	10.0%	of J	63.8
N	Buildings	6.8%	of J	43.4
O	Structures	4.8%	of J	30.6
P	Fireproofing	5.6%	of J	35.7
Q	Electrical	11.3%	of J	72.1
R	Painting & cleanup	5.6%	of J	35.7
S	Sum of material and labor			1,610.0
T	Overheads	30.0%	of S	484.0
U	Total erected costs			2,100.0
V	Engineering fee	10.0%	of U	210.0
W	Contingency fee	10.0%	of U	210.0
	Total investment			**2,520.0**

(*) factors include both material and labor contributions

Table 2. Fixed capital for major equipments of the upstream and fermentation sub-sections

Acknowledgements

The authors thank the Ministero dello Sviluppo Economico for their financial support at the project EuroTransBio ETB-2012-16 OPTISOLV (Development, optimization and scale-up of biological solvent production).

4 BIOMASS PRETREATMENT

Pretreatment of lignocellulosic materials is mandatory in the biomass-to-butanol conversion production. The objective of the pretreatment is to render cellulose and hemicellulose accessible to both chemical and enzymatic hydrolysis for efficient bioconversions. This section reports the investigation carried out at the University of Western Ontario regarding the use of a new class of solvents DES (Deep Eutectic Solvent). The activity regarded the pretreatement of corncob - a byproduct of corn grain production – to produce fermentable sugars for butanol production..

4.1 Deep Eutectic Solvent Pretreatment and Saccharification of Corncob

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Lignocellulosic biomass is a potential raw material for biofuels production. It is characterized by a complex and rigid structure that makes it highly resistant to biological and chemical degradation into monomeric sugars. This paper reports results about the pretreatment of corncob by new class of solvents: deep eutectic solvents (DESs). Tests were carried out adopting three different DES mixtures: choline chloride and glycerol; choline chloride and imidazol; choline chloride and urea. The pretretated corncob and the DES-treated corncob were characterized in term of lignin content, sugars concentration, enzymatic digestibility and crystallinity index. Choline chloride and glycerol mixture was the best DES: up to 98% glucose and xylose enzymatic conversion in 10 h. Choline chloride-glycerol DES mixture is inexpensive, non toxic, biocompatible and effective for pretreatment of corncob resulting in a fast saccharification of both cellulose and hemicellulose.

Introduction

The sustainable production of liquid fuels from renewable biomass is of academic and industrial interest (Saha et al., 2005; Jung et al., 2013; Gao et al., 2014). However, the cost of the substrate in a fermentation process is about 60% of the overall production cost. Hence, feedstock availability at high mass rate, constant rate over the year and available at low cost are key issues for the success of the biofuel production by the biotechnological route. Lignocellulosic biomass has the potential to meet these requisities even though pretreatment is required. Different pretreatment methods (physical, chemical, biological) have been well investigated for biofuel production from lignocellulosic biomass (Kumar et al., 2009; Mussatto and Teixeira, 2010). The aim of the pretreatment is to increase the accessibility of cellulose and hemicelluloses in order to obtain fermentable monomeric sugars (Galbe and Zacchi, 2007).

In the recent years various studies have focused on a particular class of solvents: ionic liquids (ILs), loosely defined as with a melting point of less than 100°C. They have low vapor pressures, high thermal stabilities and some of them are considered environmentally friendly solvents (Shill et al., 2010; Ghandi, 2014). Their ionic nature allows ILs to dissolve combinations of organic and inorganic compounds

improving the performances of diverse types of separation processes. Potential advantages have also been highlighted for the pretreatment of lignocellulosic biomass by ILs (Fu and Mazza 2011; Gräsvik et al., 2014). The inter- and intra-molecular hydrogen bonds of cellulose are thought to be disrupted and replaced by hydrogen bonding between the IL anion and the carbohydrate hydroxyls (Remsing et al., 2006). ILs have unique solubilisation characteristics, by comparison, more conventional chemical pretreatment technologies which require longer saccharification reaction times and higher enzyme amount to achieve high sugar yields. Many of these conventional chemical pretreatments produce molecules that inhibit the fermentation process (Kumar et al., 2009). Although these solvents are particularly able to solubilize lignocellulose without significant production of inhibitors, theirs commercial viability is hindered by several issues: i) costs; ii) energy demand to recycle the solvents; iii) toxicity; iv) high viscosity.

A interesting family of ionic fluids is rapidly emerging: the Deep Eutectic Solvents (DESs). DES is a fluid generally composed of two or three cheap and safe components that are capable of self-association to form a eutectic mixture. Two salts with a high melting point are mixed to obtained a liquid phase with a melting point lower than that of each individual components (Hayyan et al., 2013; Dominguez de Maria, 2013; Dai et al., 2013). These DESs exhibit similar physico-chemical properties to ionic liquids, they are environmentally friendlier and much cheaper than ILs (Gorke et al., 2008). Cost data reported so far are: ~ 65 $US/Kg for choline chloride, ~ 20 $US/Kg for urea, ~ 35 $US/Kg for glycerol vs. ~ 240 $US/Kg for 95% pure 1-butyl-3-methylimidazoliumchloride (one of the most used IL). For these reasons, more recently, deep eutectic solvents have been proposed as potential alternatives for lignocellulose biomass dissolution (Zhao et al., 2012; Xia et al., 2014). Furthermore, Leman et al., (2014) have reported that DESs have not negative effect on enzyme activity.

Potential lignocellulose biomass are agricultural residues, municipal solid waste, and dedicated energy crops. Corncob - a byproduct of corn grain production - is currently being used as a potential feedstock for cellulosic ethanol production in the United State because it is charcaterized by low lignin and high carbohydrate contents. Moreover, corncob is charcaterized by high heating value (HHV) of avout 8000 Btu/lb (Roth and Gustafson, 2014).

This papar reports results of biomass pretreatment tests carried out with DESs. The biomass tested was cornocob. The selected DESs were: choline chloride and glycerol; choline chloride and urea; choline chloride and imidazol. Two antisolvents (water and ethanol) were tested to improve the biomass recovery after pretreatment. The aim of the pretreatment was to increase the access to cellulose and hemicellulose by enzymes to produce fermentable monomeric sugars. Raw biomass and pretreated biomass were characterized in terms of lignin content, sugars concentration, inhibitors content and enzymatic digestibility.

Materials and methods

Materials

Corncob was from local farmers in Chatham, ON, Canada. It was dried and then was processed by a IKA Microfine grinder (MF10, Sigma Aldrich, Wilmington, NC) equipped with a 0.5 mm screen.

Chemicals (e.g., choline chloride, glycerol, imidazol, urea) were purchased from Sigma–Aldrich (USA).

The Cellic CTec2 cellulose enzyme from Novozyme (Canada).

DES

The tested DESs were:

a) choline chloride and glycerol, molar ratio 1:2;
b) choline chloride and urea, molar ratio 1:2;
c) choline chloride and imidazole, molar ratio 3:7.

The features of of the mixtures are reported in table 1.

DES	T_m (°C) Melting point	Tf (°C) Freezing Point	Density (g cm^{-3})	Viscosity (cP)	Conductivity (mS cm^{-1})
ChCl-Urea 1:2	134	12	1.25	750 (25°C) 169 (40°C)	0.199(40°C)
ChCl-Glycerol 1:2	17	-40	1.18	379 (20°C) 259 (25°C)	1.05 (20°C)
ChCl-Imidazol 3:7	89	56		15 (70°C)	12 (60°C)

Table 1. DES properties (Zhang et al., 2012)

Product characterization

The crystalline index (CrI) of the recovered biomass was characterized by X-ray diffraction (XRD) by means of a Rigaku (USA) equipped with the Cokα radiation source. Solid samples were milled and sieved by a 150 μm-mesh screen. Samples were scanned at a speed of 5°(2θ)/min for the continuous run in the 5 to 45° (2θ) range. The CrI was assessed according to the relantionship:

$$CrI = \frac{I_{MAX} \times I_{MIN}}{I_{MAX}} \times 100\% \quad (1)$$

where I_{max} is the maximum intensity peak for cellulose I at 2θ of about 25°, I_{min} the minimum intensity peak for cellulose II at 2θ of about 20° based on Bragg's law conversion from the CuKa radiation source.

Glucan, xylan and lignin content of the samples were measured by quantitative saccharification upon acid hydrolysis. The standard NREL procedure (Determination of structural carbohydrates and lignin in biomass, 2008) was adopted.

The concentration of glucose and xylose was measured by an Agilent 1260 Infinity high-performance liquid chromatography (HPLC) fitted with a Hiplex H Plus Column (Agilent Technologies, USA), equipped with a refractive index detector. The column temperature was set at 85°C and the flow rate was 0.4 mL min^{-1} (5mM H_2SO_4). Before analysis, hydrolyzed liquid samples were filtered through a 0.2mm cellulose acetate membrane (VWR International, USA).

Lignin contente was assessed by gravimetric analysis.

Total solids were assessed according to the standard NREL procedure (Determination of total solids in biomass and total dissolved solids in liquid process samples, 2008).

Enzymatic hydrolysis

Enzymatic hydrolysis of pretreated corncobs was carried out in 100mL glass bottles with the Cellic CTec 2 enzyme. The enzyme activity was 166 FPU/ml. The enzyme to biomass ratio (mass basis) was set at 120 µL/g. The hydrolysis mixture was 6% (w/v) dry matter/buffer. 0.1M sodium citrate buffer (pH 4.8) was supplemented with 80 µL tetracycline and 60 µL cycloheximide to prevent microbial contamination during digestion. All bottles were incubated at 50°C in a rotary shaker (Infors HT, Switzerland) at 180 rpm until 80 h. 1mL of an aliquot sample was withdrawn from each reaction mixture at different hydrolysis times, filtered and transferred to a HPLC vial for glucose analysis.

Procedure

Figure 1 shows the sinoptic diagram adopted for the corncob pretreatment by DES. After the pretreatment step, the biomass was recovered with water or ethanol. The processed biomass was used for:

i) biomass characterization to obtain the concentration of sugars, the Acid Insoluble Lignin (AIL), the Acid Soluble Lignin (ASL), the concentration of inhibitors (acetic acid, HMF, Furfural);

ii) XRD analysis aimed at the assessing the biomass structure modification;

iii) enzymatic hydrolysis to produce fermentable sugars.

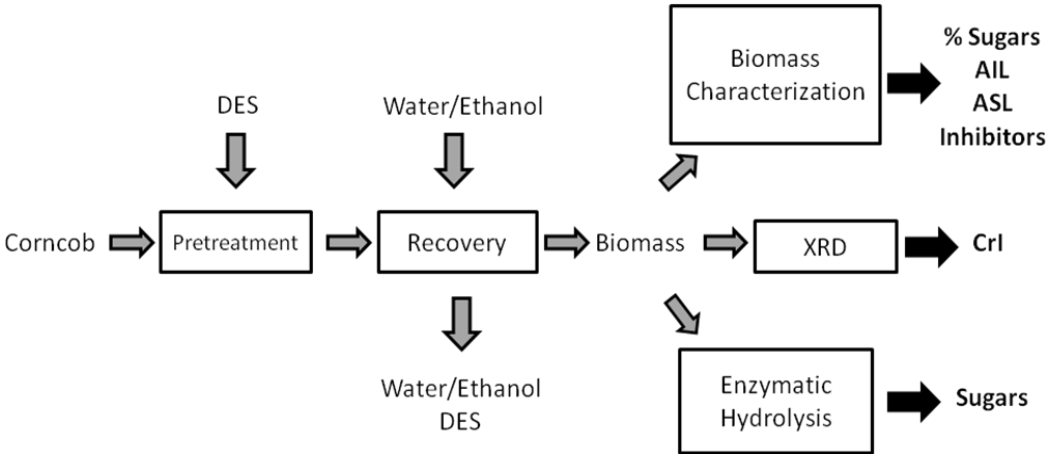

Figure 1. Sinoptic diagram of corncob pretreatment by DES.

The mixtures of selected solids solutions were stirred at 500 rpm in oil bath untile a homogeneous liquid was formed.

Corncob was add to the DES at solvent/solid ratio 16:1. The slurry was stirred for 15h. The tests were carried out at 80, 115 and 150°C. The biomass-DES slurry was washed with an antisolvent (water or ethanol) and then centrifugated. The slurry washing was repeated until DES was completely removed.

The recovered solids was dried in oven at 38°C until constant weight.

Tests were carried out in duplicate.

Results and discussion

Recovery and X-ray diffraction

The pretreated biomass was recovered with water and ethanol.

Figure 2 shows the recovered fraction of biomass pretreated with DES at three value of temperature. Both antisolvents (water and ethanol) were investigated.

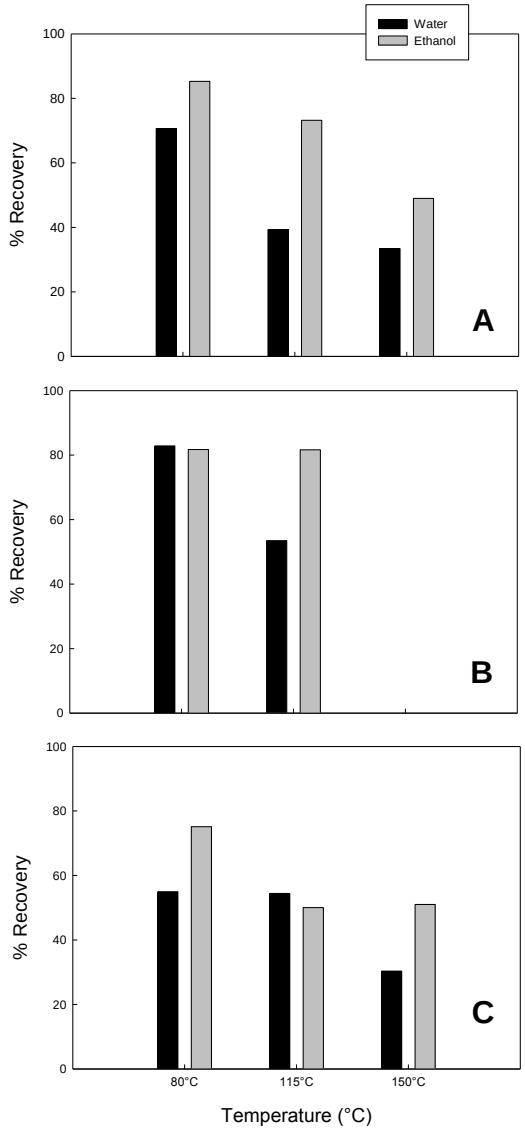

Figure 2. Biomass recovery with antisolvents after DES pretreatments: A) Ch-Cl Glycerol B) Ch-Cl Urea C) Ch-Cl Imidazol at 80°C-115°C-150°C.

Tests carried out with Ch-Cl glycerol (Fig.2A) pointed out that the recovered biomass was higher when ethanol was used than the water was used, fao all temperature. The Ch-Cl urea pretreatment (Fig.2B) was characterized by singular behaviour: at 80°C the recovered biomass was constant with the antisolvent; at 115°C the recovered biomass increased of about 40% when ethanol was used as antisolvent; at 150°C the pretreated corncob looked like a sticky paste and it was not possible further processing. The Ch-Cl imidazol pretreatment (Fig.2C) was characterized by: biomass recovery of about 50% at 115°C for both antisolvents, recovery improving moving from water to ethanol at 80 and 150°.
The comparison of these results with data reported in the literature is complex by the different type of investigated biomass. Moreover to the authors knowledge, only few studies are reported in literature regarding DESs as pretreatment tool for corncob. The comparison is hereinafter reported with results of pretreatment tests carried out with the ionic liquids, being physico-chemical features very close each other. Data regarding IL pretreatment of bagasse are by Kimon et al. (2011, 2012). The paper

focused on bagasse pretreatment with three imidazolium-based ionic liquids at high temperature (110-160°C) and the biomass was recovered with water and ethanol as antisolvent. The sticky-paste formation at high temperature was reported by Kimon et al. (2011) according to the present observation at 150°C for the Ch-Cl-urea pretreatment. Results of Kimon and co-workers confirmed the increase of recovered biomass at 150°C when moving from water (about 30%) to the ethanol (about 50%).
The structural modification of biomass after DES pretreatment was assessed by using X-ray powder diffraction (XRD) experiments. Figure 3 reports X-ray diffraction diagram of untreated corncob, pretreated corncob with Ch-Cl Glycerol at 80°C, pretreated corncob with Ch-Cl Glycerol at 150°C. X-ray diffraction diagram of pretreated corncob with Ch-Cl Glycerol at 80°C and 115°C showed the same trend (data not reported). The XRD patterns for each pretreated biomass showed an increased intensity of the diffraction peak corresponding to cellulose I. The highest increase of the diffraction peak was recorded for corncob pretreated at 150°C with Ch-Cl glycerol. The diffraction peak due to cellulose I assessed for Ch-Cl Glycerol pretreated biomass at 150°C increased of about 30% with respect to the untreated biomass peak.

Biomass characterization

Pretreated biomass was characterized in term of sugars and inhibitors content. Table 2 reports the composition of the ptretreated biomass. Glucan, xylan and arabinian content (percentage of recovered dry matter) were assessed according to the NREL protocol. Data refer to all investigated DESs and the selected temperature (see previous section), The glucan content of pretreated biomass with Ch-Cl Glycerol at 80°C-115°C and Ch-Cl Urea at 80°C-115°C was similar to sugar content for untreated biomass. The glucan content of Ch-Cl imidazol pretreatment increased with the temperature. The highest glucan content was measured with Ch-Cl Glycerol at 150°C and it was 52.7%. The xylan and arabinan content decreased with the temperature for all investigated DESs. The Acid Insoluble Lignin (AIL) content decreased with the pretreatment temperature. The Acid Soluble Lignin (ASL) content was almost constant with the temperature.
Results may be compared with those reported by Kimon et al. (2011) for tests carried out with ILs. Of course, the comparison must take into account that Kimon et al. (2011) processed bagasse with 1-butyl-3-methylimidazolium chloride at 110°C and 160°C. They found that the glucan content increased and the xylan, arabinan and AIL content decreased with the temperature.
Table 2 reports acetic acid, HMF and furfural concentrations detected after DES pretreatment. It is interesting to compare the values reported in Table 2 with data reported by Baral and Shah (2014). The review summarized the current knowledge regarding the formation of microbial inhibitors during the most common lignocellulosic biomass pretreatments. Process toxicities for hydrothermal, steam explosion, sulphuric acid and oxalic acid pretreatments was reported. Acetic acid concentration was between ≈ 0 g L^{-1} (for hydrothermal pretreatment) and 5 g L^{-1} (for sulphuric acid pretreatment). The acetic acid concentration for the DES pretreatments (less than 0.2 g L^{-1} for all DESs) was at the lower limit of the identified range. The HMF concentration was in a range 0.1 g L^{-1} (for hydrothermal pretreatment) to 1 g L^{-1} (for sulphuric acid pretreatment). No HMF was detected after Ch-Cl glycerol and Ch-Cl urea pretreatments.

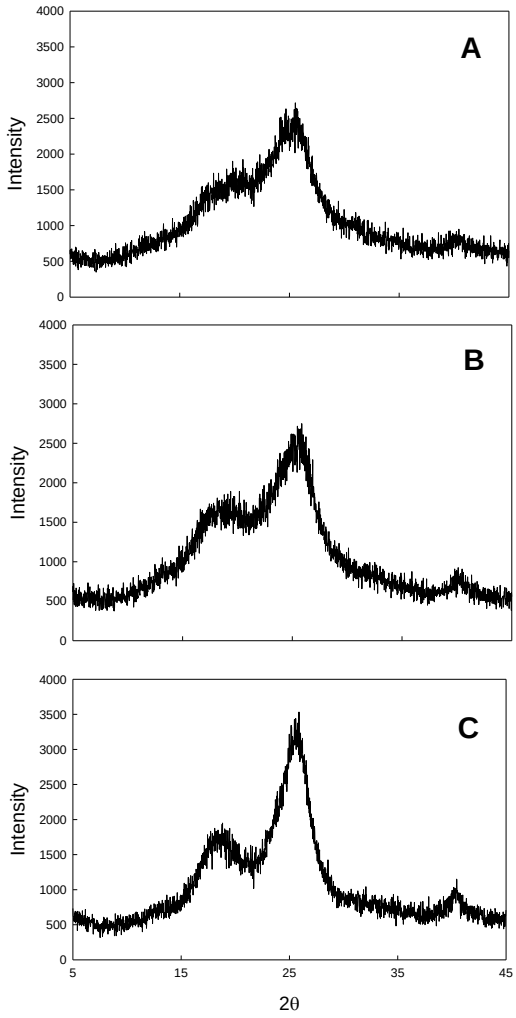

Figure 3. X-ray diffraction diagram of untreated and pretreated corncob. A) unpretreated corncob; B) Pretreated corncob with Ch-Cl Glycerol 80°C; C) Pretreated corncob with Ch-Cl Glycerol 150°C

Baral and Shah reported the furfural concentration ranging between 0.5 g L^{-1} (for steam explosion pretreatment) and 5 g L^{-1} (for sulphuric acid pretreatment). The furfural concentration for the DES pretreatment (less than 0.2 g L^{-1} after all DES pretreatment) was definitevily lower than the reported inhibitory levels for typical yeast strains (DOI 10.1007/s12155-014-9535-4).

Pretreatment	Composition[a] (%w)					Inhibitors (g L^{-1})		
	Glucan	Xylan	Arabinan	AIL	ASL	Acetic Acid	HMF	Furfural
Unpretreated	31.5± 1.2	22.1±0.4	3.1±0.3	13.7±0.1	3.2±0.1			
Ch-Cl Glycerol 80°C	32.5±2.4	25.3±1.9	3.3±0.1	13.1±0.3	2.7±0.1	0.18±0.01	-	0.14±0.01
Ch-Cl Glycerol 115°C	31.9±1.2	24.3±1.2	2.9±0.2	12.5±0.8	2.7±0.2	0.14±0.03	-	0.12±0.01
Ch-Cl Glycerol 150°C	52.7±1.2	21.1±1.1	-	10.3±	2.3±0.1	0.13±0.01	-	0.11±0.01
Ch-Cl Urea 80°C	33.3±1.6	26.9±1.5	3.9±0.2	12.6±3.1	2.6±0.1	0.16±0.02	-	0.12±0.02
Ch-Cl Urea 115°C	32.4±0.1	23.7±0.4	3.3±0.3	10.3±0.4	2.9±0.3	0.09±0.01	-	0.12±0.02
Ch-Cl Imidazol 80°C	38.4±2.8	30.0±1.8	4.6±0.7	7.8±0.1	2.9±0.1	0.01±0.03	0.09±0.01	0.04±0.01
Ch-Cl Imidazol 115°C	46.2±4.2	22.3±3.2	2.6±0.2	4.0±0.5	2.8±0.2	0.03±0.01	0.06±0.02	0.04±0.01
±**Ch-Cl Imidazol 150°C**	41.1±3.6	5.6 ±0.5	-	1.6±0.5	2.8±0.1	0.08±0.01	0.02±0.01	0.01±0.03

[a] Results are expressed as a percentage of the pretreated biomass, oven-dried basis.

Table 2. Comparison of various DES pretreatments for corncob. Each test was carried out in duplicate and the reported results are the mean values.

Enzymatic hydrolysis

Figure 4 rports the time course of the enzymatic hydrolysis of the DES pretreated biomass with Cellic CTec2 at 50°C. The initial rate of hydrolysis and the final amount of sugars were enhanced by the DES pretreatment. As reported in Fig. 4 to saccharify the cellulose took a much longer time period (approximately 50 h) for the biomass pretreated at 80°C than those pretreated at 115°C (approximately 20 h) or at 150°C (approximately 10 h).

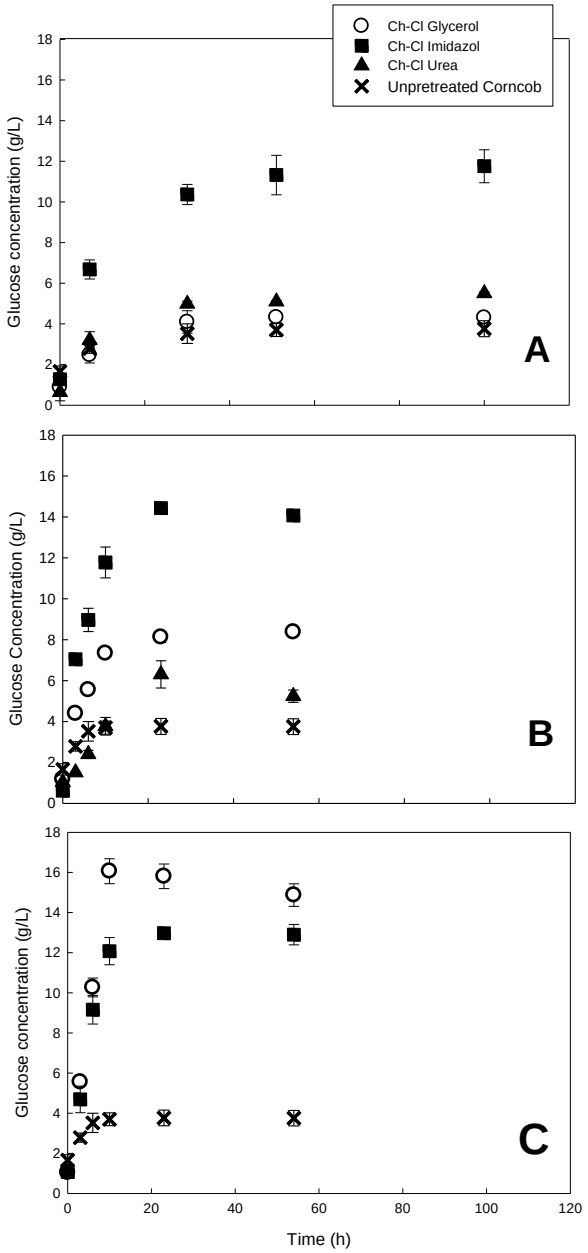

Figure 4. Enzymatic hydrolysis of unpretreated and pretreated corncob by DES at A)80°C B)115°C C)150°C. Each test was carried out in duplicate and the reported results are the mean values. The error barrs indicate the deviation standard between the duplicates.

Similar results for xylan digestibility were obtained (data not reported).
Table 3 reports the crystallinity index (CrI), cellulose digestibility and xylose yields for all DES pretreatments. The CrI was calculated according to Eq.(1) and XRD data. It can be noticed that the CrI and the cellulose digestibility increased with the

pretreatment temperature for all DES. As regard xylose, the maximum xylose yield was obtained with Ch-Cl glycerol at 150°C .

Pretreatment	Cr Index	Cellulose digestibility[a](%)	Xylose Yields[b] (%)
Unpretreated	30.07	32.8	15.5
Ch-Cl Glycerol 80°C	31.20	39.9	17.7
Ch-Cl Glycerol 115°C	29.79	79.1	61.3
Ch-Cl Glycerol 150°C	44.81	91.5	95.5
Ch-Cl Urea 80°C	23.62	51.0	22.9
Ch-Cl Urea 115°C	36.54	58.6	31.2
Ch-Cl Imidazol 80°C	31.58	92.3	59.5
Ch-Cl Imidazol 115°C	40.08	94.0	84.0
Ch-Cl Imidazol 150°C	49.22	94.6	84.8

[a] Results are expressed as a percentage of grams cellulose digested on grams cellulose add.
[b] The mass of xylose released via enzymatic hydrolysis divided by the mass of xylose in the pretreated biomass.
Table 3. Comparison of CrI (obtained from XRD), cellulose digestibility and xylose yields (obtained from enzymatic hydrolysis).

The analysis of table 3 points out that the cristallinity index increased with the cellulose digestibility. This results is particularrly interesting when compared with results by Fu and Mazza (2011). They reported that the cellulose digestibility decreased when the cristallinity index increased. However, enzymatic cellulose hydrolysis is a complex process and CrI alone may not explain the observed hydrolysis rate. As reported by Park et al. (2010) the correlation between amorphous regions - easy to digest - and crystalline regions - difficult to digest - is appealing even though the cellulose hydrolysis by enzymes may be governed by different features: available surface area, degree of polymerization, particle size, hemicellulose removal. Furthermore, as reported in the literature (Kim and Holtzapple, 2006; Zheng et al., 2013) a biomass with high crystallinity index does not necessarily negatively affect the enzymatic hydrolysis rate.
Table 4 reports the comparison of the results of the DES pretreatment with typical pretreatment reported in the literature focused on corncob. Table 4 compares the yields (g/g) - defined as the ratio between the obtained sugars and the grams of initial biomass - resulting from enzymatic hydrolysis of corncob with different pretreatment methods. All experiments were conducted using adequate enzyme loading, which provide good indication of the individual pretreatment efficiency. As reported in Table 4 the highest yield is reached by steam explosion pretreatment. Similar values are reported for DES, IL and Na-OH pretreatments. A complete comparison should take into account energetic and environmental issues.

Feedstock	Pretreatment	Yield (g/g)	References
Corncob	Untreated	0.10	This study
Corncob	DES	0.73	This study
Corncob	IL	0.53*	Bahcegul et al. (2011)
Corncob	Sodium Hydroxide	0.80	Potumarthi et al. (2014)
Corncob	Sulfuric acid	0.11	Potumarthi et al. (2014)
Corncob	Steam-explosion	1.20	Fan et al. (2014)

*The value refers to glucose only.
Table 4. Comparison of different pretreatments of corncob.

Conclusion

DES (choline chloride-glycerol) was assessed as inexpensive, non toxic, biocompatible pretreatment of corncob resulting in a fast saccharification of both cellulose and hemicellulose. For choline chloride-glycerol pretreatment at 150°C the cellulose digestibility reached 91.5% and the enzymatic hydrolysis took 10h to reach 15g L^{-1} of glucose. This study showed great potential for industrial application as efficient pretreatment to obtain fermentable sugars from corncob.

5 FERMENTATION PROCESS

This section is about the activity carried out to characterize the ABE fermentation process as regards kinetics and yields. The attention was focused on both the acidogenesis and the solventogenesis phases. Accordingly, two typologies of reactor systems were used: a CSTR equipped with a pH controller, and an CSTR equipped with a pH controller and a microfiltration unit. Tests with the first reactor were aimed at the characterization of the fermentation process under acidogenesis conditions. Tests with the second reactor were aimed at the characterization of the solventogenesis phase.
The last subsection reports a potential application of the kinetic characterization. The successive steps for the development of a simulation model for ABE fermenter systems are highlighted.

5.1 Continuous xylose fermentation by Clostridium acetobutylicum – Assessment of acidogenesis kinetics

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The aim of the present paper is an assessment of the growth kinetics of *Clostridium acetobutylicum* DSM 792. A complex medium supplemented with xylose, one of the mains components of the lignocellulose hydrolysates, was used The effects of acids (acetic and butyric) and solvents (acetone, ethanol and butanol) on the growth rate of acidogenic cells has been investigated. Tests were carried out in a CSTR operated under controlled pH. The conversion process was characterized under steady-state conditions in terms of concentration of xylose, cells, acids and pH. The growth kinetics was expressed by means of a multiple product inhibition.

Introduction

The impact of petroleum fuel emissions and more rapid diminishing petroleum reserves have increased the research for alternative biofuel sources (Van Hecke et al., 2012). Butanol is a ideal alternative fuel. It offers several advantages over ethanol: high energy content, low miscibility with water, and low volatility (Bohlmann, 2007; Cascone, 2008). In addition, butanol can be mixed in higher ratios with gasoline for use in existing cars without modifications in gasoline-designed engine. n-butanol is currently produced on a scale of 5–6 million tons per year, with a worldwide market sale of US$7–8.4 billion (Cheng et al., 2012). In this context, acetone–butanol–ethanol (ABE) fermentation is being considered as a way to upgrade renewable resources into liquid fuels (Ezeji et al., 2007a; Dürre, 2007). ABE is typically produced during the last stage of batch fermentation of some Clostridium strains (*C. acetobutylicum, C. beijerinckii, C. aurantibutyricum*). During the fermentation of Clostridia two separate growth phases occur: the acidogenic phase and the solventogenic phase. During the acidogenic phase cell grow and produce acids and gas: butyrate (butyric acid) and acetate (acetic acid), carbon dioxide and hydrogen. The production of the acids causes a decrease in the pH, therefore

environmental conditions are no more favourable to bacterial growth. *Clostridium acetobutylicum* responds to this new conditions with a metabolic and morphological shift: i) the exponential growth phase ends; ii) the active cells become endospores, unable to grow; iii) the acids are converted to solvents, Acetone-Butanol-Ethanol (in a typical molar ratio 3:6:1); iv) the substrate is also converted directly in solvents, Acetone-Butanol-Ethanol. The fermentation process ends because high concentration the solvents inhibit the process: cell membranes are solubilized and cell death. (Jones and Woods, 1986). The traditional batch fermentation process for butanol production is characterized by 3 major issues: the low product concentration and productivity in fermentation due to end-product inhibition (Zeng et al., 1994), the high product recovery cost (Napoli et al., 2012a), the high cost of the substrate which represents about 60% of the overall production cost. Increased productivity was achieved operating in continuous mode and increasing cell concentration by cell recycling or immobilization (Qureshi et al., 2008-2013; Raganati et al., 2013). Studies on fermentation and simultaneous product recovery to decrease the product inhibition effects are be carried out (Wu et al., 2012; Ezeji et al., 2012). As regard the substrate, it has been paid much attention on fermentation from renewable resources as lignocellulosic materials. Indeed, wood and agricultural residues are cheap, sustainable and more abundant resources (Jang et al., 2012). They consist mainly of cellulose and hemicellulose and different Clostridium strains are able to metabolize pentoses and hexoses (Flickinger and Drew, 1999). Different studies have focused on butanol fermentation with xylose as the sole carbon source. Shinto et al.(2008) reported, higher yield of butanol from xylose (0.62 C-mol/C-mol) than from glucose (0.53 C-mol/C-mol) in batch culture, suggesting xylose is a useful substrate for ABE fermentation. Zheng et al. (2013) achieved maximum butanol productivity 3.32 g L^{-1} h^{-1} with continuous fermentation on medium supplemented with xylose.

Furthermore, studies on continuous production of solvents by clostridia strains report the simultaneous production of acids and solvents suggesting that acidogenic and solventogenic cells coexist in the cultures (Huang et al., 2004; Qureshi and Blaschek, 2005; Ezeji et al., 2007a; Napoli et al., 2009). The simultaneous presence of both acidogenic and solventogenic cells make the ABE fermentation a very complex system to be investigated. To improve the overall process is necessary to know the growth kinetics of *Clostridium acetobutylicum*.

The kinetics of acidogenic cell growth in a CSTR operated under controlled pH is the focus of the present paper. The effects of acids and solvents on the growth rate of *C. acetobutylicum* cells under acidogenesis phase conditions has been investigated. The study aims at providing kinetic models and parameters for butanol production adopting a medium supplemented with xylose. A specific procedure has been proposed to assess model parameters. Further the aim will be to extend the model to describe cell growth rate also under solventogenesis conditions.

Materials and methods

Microorganism and media

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Stock cultures were reactivated according to the method suggested by the supplier and stored at -80°C. The cells were inoculated into 12 mL synthetic pre-culture medium containing: 5 g L^{-1} Yeast Extract , 2 g L^{-1} ammonium chloride (nitrogen source), 30 g L^{-1} glucose(carbon source) ,0.5 g L^{-1} KH_2PO_4- 0.5 g L^{-1} K_2HPO_4 (buffer), 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.01 g L^{-1} $MnSO_4.7H_2O$, 0.01 g L^{-1} $FeSO_4.7H_2O$ (minerals). Prior to the addition of the carbon source, the buffer and the minerals, the medium was sterilized

in autoclave (121 °C, 20min). The feeding medium had the same composition of pre-culture medium except for the carbon source which was 40 g L^{-1} xylose. Chemicals and yeast extract were from Sigma Aldrich.

Apparatus

Continuous fermentations were carried out at 37°C in 1 L vessel (Pyrex®) containing 0.5 L medium Fig.(1). Nitrogen was sparged at the bottom of the reactor to preserve the anaerobic and stirred condition. The gas stream was sterilized by filtration (cut-off 0.2 µm, Millipore). Inlet liquid stream was handled by a peristaltic pump (Gilson Minipuls 3). The liquid phase volume in the reactor, was modified by changing the level of the overflow duct. The reactor was equipped with a pH and temperature controller. pH was keep at the desired value by a controller (Applikon Bio Controller ADI 1030) equipped with 1 M NaOH solution tank. Temperature was controlled by a water jacket connected to a thermostatic water bath. The reactor vessel and the medium were sterilized in autoclave.

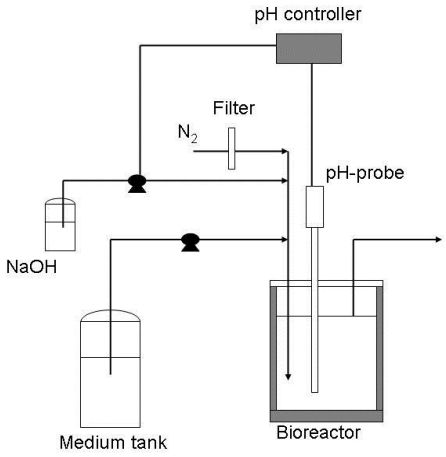

Figure 1. Outline of the apparatus adopted for continuous test.

Analytical methods

Cell density was measured as optical absorbance at 600 nm (OD600) using a spectrophotometer (Cary- 50 Varian). Calibration tests for *C. acetobutylicum* dried mass indicated that 1 OD600 = 0.4 g_{DM} L^{-1}. pH was also measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments). The concentration of soluble species was measured in the liquid phase after centrifugation (11,000g @ 10min). Sugar concentration was measured by high performance liquid chromatography (Agilent 1100 system). Deionized water was used as mobile phase at 0.6 mL min^{-1} flow rate. The sugars were separated on a 8 µm Hi-Plex H, 30 cm x 7.7 mm at room temperature and detected with a refractive index detector. Metabolite concentrations were measured by means of a GC apparatus (Agilent 7890A) equipped with a FID, and outfitted with a capillary column poraplot Q (25 m x 0.32 mm). Internal standard (hexanoic acid) was adopted to assess acids and alcohols concentrations.

Operating conditions and procedures

300 µL of stock culture were transferred into four 15-mL Hungate tubes containing the pre-culture medium (30 g L^{-1} of glucose). The pre-cultures were

incubated for 2 day, then 40 mL of active culture were inoculated in the reactor containing 0.5L feeding medium. Typically, after 6 h of batch culture the xylose-bearing stream was fed to the reactor at the desired dilution rate (D). Tests were carried out at 37 °C under anaerobic conditions. The set-point for the pH controller was fixed at value 5.5. The dilution rate was changed between 0.1 and 0.9 h^{-1}. The culture was periodically sampled to measure biomass and metabolite concentrations until steady-state conditions were reached. 4–5 times the liquid space–time were attended.

Theoretical framework and design of the experiments

Growth rate model

The growth kinetics of *C. acetobutylicum* is characterized by products inhibition. In agreement with Han and Levenspiel (1988) the kinetic models, with reference to single-product inhibited growth, typically conform to one of the following expressions:

Aiba-type

$$\mu = \mu_{\max} \frac{S}{S + K_S} e^{-P/Kp} \quad (1)$$

Ierusalimsky-type

$$\mu = \mu_{\max} \frac{S}{S + K_s} \frac{1}{1 + P/K_P} \quad (2)$$

Luong-type

$$\mu = \mu_{\max} \frac{S}{S + K_S} \left(1 - \frac{P}{P_{\max}}\right)^n \quad (3)$$

Model (3) better describes the behaviour of *C. acetobutylicum*. The cell growth is characterized by full inhibition as the concentration of inhibitor metabolites approaches a critical value (P_{max}). The growth kinetics of *C. acetobutylicum* under acidogenesis conditions at a fixed pH can be described, in agreement with Napoli et al. (2011), by the interactive multiproduct-inhibited model (4) :

$$\mu = \mu_{\max} \cdot \frac{Xyl}{Xyl + K_{Xyl}} \cdot \left(1 - \frac{AA}{AA_{\max}}\right)^{n_{AA}} \cdot \left(1 - \frac{BA}{BA_{\max}}\right)^{n_{BA}} \cdot \left(1 - \frac{Ac}{Ac_{\max}}\right)^{n_{Ac}} \cdot \left(1 - \frac{Et}{Et_{\max}}\right)^{n_{Et}} \cdot \left(1 - \frac{B}{B_{\max}}\right)^{n_B} \quad (4)$$

where μ_{max} is the maximum specific growth rate, K_{Xyl} the Monod coefficient for xylose, μ under steady-state conditions is the dilution rate (D) and Xyl, AA, BA, Ac, Et, B the concentration of xylose, acetic acid, butyric acid, acetone, ethanol and butanol, respectively. Acidogenic and solventogenic cells coexist in the cultures hence there is the need to describe the kinetics of acidogenic cells even during solvent production. Due to the high number of parameters to be determined (μ_{max}, K_X, AA_{max}, BA_{max}, Ac_{max}, Et_{max}, B_{max}, n_{AA}, n_{BA}, n_{Ac}, n_{Et} and n_B), a multistep parameter inference procedure was required to infer the kinetic parameters.

Step (1) – Preliminary estimate of μ_{max}, K_X, AA_{max}, BA_{max}: (μ'_{max} , K'_x, AA'_{max}, BA'_{max}) were obtained by a best-fit procedure applied to data obtained during tests carried out at dilution rates D between 0.1 and 0.9 h^{-1} at 40 g L^{-1} xylose concentration in the feeding. Under these conditions, solvents concentration was negligible. Moreover, in agreement with Napoli et al.(2009) the molar ratio between butyric acid and acetic acid concentration is constant and equal to 1.5. So, assuming $n_{AA} = n_{BA} = 1$ Eq. (4) was reduces to:

$$\mu = \mu_{\max} \cdot \frac{Xyl}{Xyl + K_{Xyl}} \cdot \left(1 - \frac{Acid}{Acid_{\max}}\right)^2 \quad (5)$$

Step (2) – Exact estimate of AA_{max}, BA_{max}, μ_{max}, K_X, n_{AA}, n_{BA}: The complete set of these parameters was determined by a parametric inference procedure applied to Eq. (6) matched against data obtained in experiments carried out with controlled addition of acids in the feeding (1 g L^{-1} acetic acid or 3 g L^{-1} butyric acid).
The preliminary estimates obtained in the previous step were used as the starting point of the multivariate best-fit convergence procedure.

$$\mu = \mu_{\max} \cdot \frac{Xyl}{Xyl + K_{Xyl}} \cdot \left(1 - \frac{AA}{AA_{\max}}\right)^{n_{AA}} \cdot \left(1 - \frac{BA}{BA_{\max}}\right)^{n_{BA}} \quad (6)$$

Step (3) – Estimation of Ac_{max}, Et_{max}, B_{max}, n_{Ac}, n_{Et} and n_B: The assessment of these parameters following Eq.(7) was accomplished by working out data measured in tests in which solvents(3 g L^{-1} butanol or 10 g L^{-1} ethanol or 20 g L^{-1} acetone) were purposely added to the feed.

$$\mu = \mu_{\max} \cdot \frac{Xyl}{Xyl + K_{Xyl}} \cdot \left(1 - \frac{AA}{AA_{\max}}\right)^{n_{AA}} \cdot \left(1 - \frac{BA}{BA_{\max}}\right)^{n_{BA}} \cdot \left(1 - \frac{Solvent}{Solvent_{\max}}\right)^{n_{SOLVENT}} \quad (7)$$

Assessment of energetics and product yields of the acidogenesis

In agreement with Bauchop and Elsden (1960) ATP yields (Y_{ATP}) is expressed as the amount of dry weight of microorganism produced per g mol of ATP. The maximum value of the ATP yield was found to range between 28.6 and 32.1 g_{DM}/molATP for microorganisms growing on glucose (Meyer et al. 1989). Pirt et al. (1965) show that the Y_{ATP} of a given microorganism may change with the specific growth rate as well as with the media composition. Eq.(8) show the relationship between YATP and the specific growth rate (μ) proposed by Pirt:

$$\frac{1}{Y_{ATP}} = \frac{1}{Y_{ATP}^{MAX}} + \frac{m}{\mu} \quad (8)$$

where m is the maintenance coefficient. Stouthamer et al. (1973) defined m "as the energy necessary for the turnover of substance and other cell components and for the preservation of the right ionic composition of the cells". Eq.(8) assumes that the consumption of energy (m) is constant and that it does not depend on the specific growth rate. Later, Pirt et al.(1982) proposed that the maintenance energy depends on the specific growth rate according to:

$$m = m_0 + m_1\left(1 - \frac{\mu}{\mu_{MAX}}\right) \quad (9)$$

where μ_{max} is the maximum specific growth rate, m0 the maintenance coefficients at μ equal to μ_{max}. Assuming m0 negligible, the combination of Eqs. (8) and (9) yields:

$$Y_{ATP} = Y_{ATP}^{MAX} \frac{\mu}{\mu + Y_{ATP}^{MAX} \times m_1\left(1 - \left(\mu / \mu_{\max}\right)\right)} \quad (10)$$

The agreement between the experimental data and the model is satisfactory and supports the validity of the Eq.(10) (Pirt et al. 1982, Napoli et al.2012b).
Furthermore, fermentation on xylose by acidogenic cells of *C. acetobutylicum* follows the stoichiometry of three main reactions:

Biomass formation

$$C_5H_{10}O_5 + \frac{5}{4}NH_3 + \frac{5}{4}\frac{MW_X}{Y_{ATP}}ATP \rightarrow \frac{5}{4}C_4H_7O_2N + \frac{5}{2}H_2O \quad (11)$$

Acetic acid formation

$$C_5H_{10}O_5 + \frac{5}{3}H_2O \rightarrow \frac{10}{3}ATP + \frac{5}{3}C_2H_4O_2 + \frac{5}{3}CO_2 + \frac{10}{3}H_2 \quad (12)$$

Butyric acid formation

$$C_5H_{10}O_5 \rightarrow \frac{5}{2}ATP + \frac{5}{6}C_4H_8O_2 + \frac{5}{3}CO_2 + \frac{5}{3}H_2 \quad (13)$$

In agreement with Napoli et al. 2012b the molar ratio between butyric and acetic acids is 1.5 so, it is possible to describe acids production with only one reaction, linear combination of Eqs. (12) and (13) :

Acids formation

$$2.4C_5H_{10}O_5 + H_2O \rightarrow 6.5ATP + C_2H_4O_2 + 1.5C_4H_8O_2 + 4CO_2 + 5H_2 \quad (14)$$

Analysis of Eq.(14) suggests that:

- the molar ratio H_2/CO_2 is 1.25
- the molar fractional yield ATP/xylose is 2.7
- According to the definition yield ATP:

$$Y_{ATP} = Y_{A/ATP} \times Y_{X/A} = 29.5Y_{X/A} \quad (15)$$

Finally, the linear combination of Eqs. (11) and (14) yields:

$$C_5H_{10}O_5 + \frac{5}{4(1+2.4\alpha)}NH_3 + \frac{\alpha}{(1+2.4\alpha)}H_2O \rightarrow \frac{5}{4(1+2.4\alpha)}C_4H_7O_2N +$$

$$\frac{\alpha}{1+2.4\alpha}C_2H_4O_2 + \frac{1.5\alpha}{1+2.4\alpha}C_4H_8O_2 + \frac{4\alpha}{1+2.4\alpha}CO_2 + \frac{5\alpha}{1+2.4\alpha}H_2 + \frac{5}{2(1+2.4\alpha)}H_2O \quad (16)$$

Where assuming that the ATP produced along with acids production is consumed for biomass formation :

$$\alpha = \frac{1.25(MW_X / Y_{ATP})}{6.5} = 19.42 / Y_{ATP} \quad (17)$$

biomass and acids fractional yields are obtained working out Eqs. (16) and (17) :

$$Y_{X/Xyl} = \frac{5/4}{(1+2.4\alpha)}\frac{MW_X}{MW_{Xyl}}g_{DM}/g \quad (18)$$

$$Y_{BA/Xyl} = \frac{1.5\alpha}{1+2.4\alpha}\frac{MW_{BA}}{MW_{Xyl}}g/g \quad (19)$$

$$Y_{AA/Xyl} = \frac{\alpha}{1+2.4\alpha}\frac{MW_{AA}}{MW_{Xyl}}g/g \quad (20)$$

Kinetic model

Table 1 reports selected results of a representative set of runs carried out with xylose at 40 g L^{-1} without add of acids or solvents. The dilution rate was changed between 0.1 and 0.9 h-1. In agreement with step (1) data reported in Table 1 were worked out for a first estimate of μ_{max} , K_{xyl} , AA_{max} and BA_{max} . The obtained values were μ'_{max} = 1.2 h^{-1}, K'_{xyl} = 0.07 g L^{-1}, AA'_{max} = 7.3 g L^{-1} and BA'_{max} = 16 g L^{-1}.

D = μ (h^{-1})	Concentration in the effluent stream		
	Acetic acid (g L^{-1})	Butyric acid (g L^{-1})	Xylose (g L^{-1})
0.08	4.5	10	0.09
0.18	4	9.0	0.1
0.30	3.3	7.5	1
0.47	2.9	6.5	6
0.65	2.1	4.8	19
0.70	1.5	3.2	23
0.85	1.0	2.1	27

Table 1. Results of tests carried out with xylose at 40 g L^{-1}

Inhibition by acids

Table 2 reports data resulting from tests carried out with the aim of determining the exact estimate of AA_{max}, BA_{max}. In agreement with step (2) μ_{max}, K_{Xyl} , the critical concentration of acids, n_{AA} and n_{BA} has been assessed: μ_{max}=1.19h^{-1}, K_{Xyl}= 0.06 g L^{-1} AA_{max}=7.12 g L^{-1} , AB_{max}=14.75 g L^{-1} ,n_{AA} = 1 and n_{BA} =0.73.

Supplemented product	D = μ (h^{-1})	Concentration in the effluent stream		
		Acetic acid (g L^{-1})	Butyric acid (g L^{-1})	Xylose (g L^{-1})
Butyric acid	0.19	3	9.5	0.08
	0.51	2	7.5	3
	0.72	0.9	5	24
Acetic acid	0.70	2	2.1	20
	0.45	3.5	5.5	8
	0.30	4.2	7	1

Table 2. Results of tests carried out with xylose at 40 g L^{-1} supplemented with 1 g L^{-1} acetic acid or 3 g L^{-1} butyric acid.

It is interesting to compare the parameter assessed in this study with data previously reported in the literature. For *C. acetobutylicum* ATCC824, on complex medium supplemented with glucose, toxic levels of acetic acid and butyric acid concentrations are of about 12 and 11 g L^{-1} respectively(Yang and Tsao, 1994). Napoli et al.(2011 determined the critical concentration of butyric acid 3.00 g/L, acetic acid 1.56 g L^{-1}, μ_{max}=1.06h^{-1} and K_L= 1.34 g L^{-1} on complex medium supplemented with lactose. As reported in fig.(2), in this study the molar ratio of acids and the μ_{max} value were in agreement with data previously reported for the lactose by Napoli et al. (2010-2012b) on the contrary the critical acids concentrations and the K_{Xyl} value are considerably different from those reported by Napoli et al. (2010). In particular the higher critical acids concentrations together with the lower K_{Xyl} value suggested a better growth rate of *C. acetobutylicum* on xylose with respect to lactose.

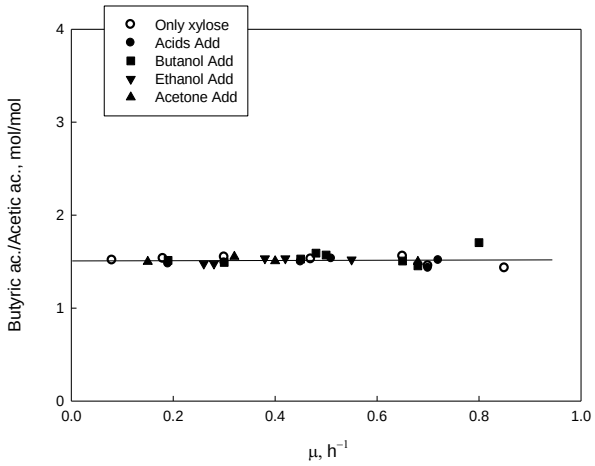

Figure 2. Molar ratio between butyric and acetic acids measured during steady state cultures under acidogenic conditions.

Inhibition by solvents

Table 3 reports data resulting from tests carried out by supplementing acetone or butanol or ethanol to the feeding medium. The solvents concentration in the feeding was set at about 1/3 the critical solvents concentration determined by Napoli et al.(2010). In agreement with Eq.(7) the critical solvents concentration was assessed.
The final results were:

$$\begin{array}{ll} Ac_{max} = 65g\ L^{-1} & n_{Ac} = 0.9 \\ B_{max} = 18g\ L^{-1} & n_{B} = 0.4 \\ Et_{max} = 38g\ L^{-1} & n_{Et} = 0.9 \end{array} \qquad (21)$$

The obtained values were in agreement with the data reported by Napoli et al. (2010). It is interesting to note that the critical acetone and ethanol concentrations were quite larger than those usually achieved during the ABE fermentation. So, the inhibitory effect of acetone and ethanol is typically negligible with respect to that of butanol. The critical concentration assessed for butanol is accordance with that typically reported in the literature (Yang and Tsao 1994, Qureshi et al., 2005; Ezeji et al., 2007b).

Overall assessment of the kinetic model

A parity plot comparing predicted values of the specific growth rate, using Eq. (4) and model parameters reported in Table 4 , with experimental values is reported in Fig. 3. Inspection of the parity plot indicates that the departure of model predictions from experimental data points is fairly small. The finding suggests that the model based on application of Eq. (4) is able to correctly reproduce the growth kinetics of *C. acetobutylicum* DSM 792 on xylose.

Energetics and products yields

Data collected during the sets of runs reported in Table (1),(2) and(3) were worked out to determine the values of Y_{ATP}, Y_{ATP}^{MAX} and m1, similarly, the same data

have been worked out to assess the fractional yields of biomass ($Y_{X/L}$), butyric acid ($Y_{BA/L}$) and acetic acid ($Y_{AA/L}$) holding during the experiments.

Supplemented product	D = μ (h^{-1})	Concentration in the effluent stream		
		Acetic acid (g L^{-1})	Butyric acid (g L^{-1})	Xylose (g L^{-1})
Butanol	0.19	4.1	9.1	0.9
	0.30	3.2	7	2
	0.45	2.9	6.5	3
	0.48	2.7	6.3	4
	0.50	2.3	5.3	10
	0.65	1.9	4.2	23
	0.68	1.5	3.2	25
	0.80	0.6	1.5	28
Ethanol	0.26	3.6	7.8	1
	0.28	3	6.5	2
	0.38	2.4	5.4	14
	0.42	2	4.5	27
	0.55	1.3	2.9	34
Acetone	0.15	4	8.8	1
	0.32	2.5	5.7	15
	0.40	1.9	4.2	26
	0.68	1	2.2	35

Table 3. Results of tests carried out with xylose at 40 g L^{-1} supplemented with 3 g L^{-1} butanol or 10 g L^{-1} ethanol or 20 g L^{-1} acetone

Yield ATP

Data reported in Table (1),(2) and(3) were worked out in agreement with Eq.(15) to determine the values of Y_{ATP}.

A best fitting procedure applied to Y_{ATP} data matched against Eq.(10), with μ_{max} = 1.19 h^{-1} yields:

$$Y_{ATP}^{MAX} = 29.6 g_{DM} / mol_{ATP} \qquad m_1 = 0.01 mol_{ATP} / g_{DM} h \qquad (22)$$

Fig.4 reported Y_{ATP} vs specific growth rate. The agreement between the experimental data and the model supports the validity of Eq.(10). The results obtained for Y_{ATP}^{MAX} and m1 are in agreement with Napoli et al. 2012b.

μ_{max} = 1.19 (h^{-1})	K_x = 0.06 (g L^{-1})
AA_{max} = 7.12 (g L^{-1})	n_{AA} = 1
BA_{max} = 14.75 (g L^{-1})	n_{BA} = 0.73
A_{max} = 65 (g L^{-1})	n_A = 0.9
B_{max} = 18 (g L^{-1})	n_B = 0.4
E_{max} = 38 (g L^{-1})	n_E = 0.9

Table 4. Model parameters of Eq.(7)

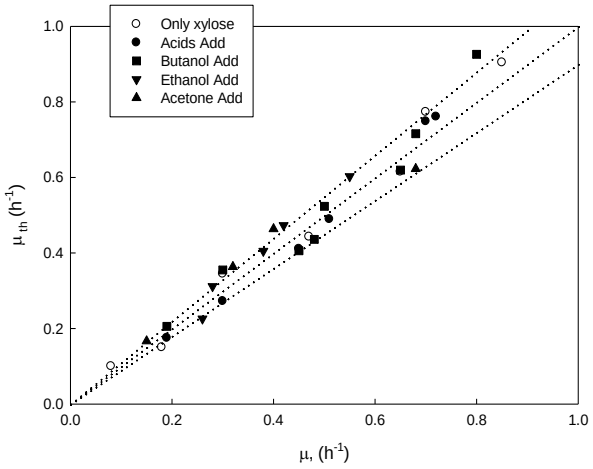

Figure 3. Measured vs. predicted specific growth rate of *C. acetobutylicum*. Crosshair : ±10% error.

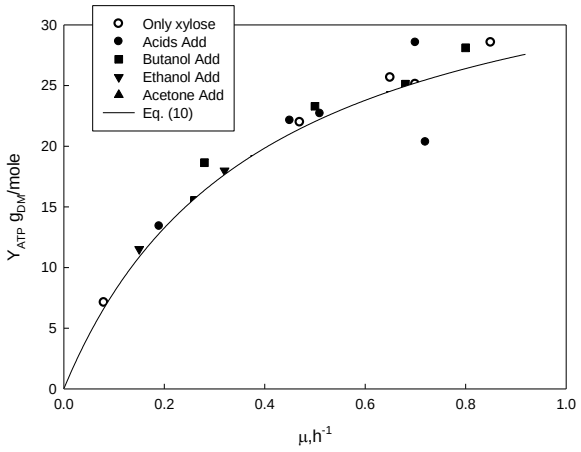

Figure 4. Y_{ATP} vs specific growth rate

Products fractional yield

Data collected in Table (1),(2) and(3) have been worked out to assess the fractional yields of biomass ($Y_{X/L}$), butyric acid ($Y_{BA/L}$) and acetic acid ($Y_{AA/L}$) holding during the experiments. Results have been reported in Fig. (5) as a function of the specific growth rate. The theoretical values of the yields were estimated according to Eqs. (18)–(20) with parameters specified in (22) and reported Fig. (5) (continuous line). As reported by Napoli et al. 2012b the agreement between the experimental data and the theoretical values confirmed the soundness of theoretical framework and the reliability of parameters reported in Eq. (22).

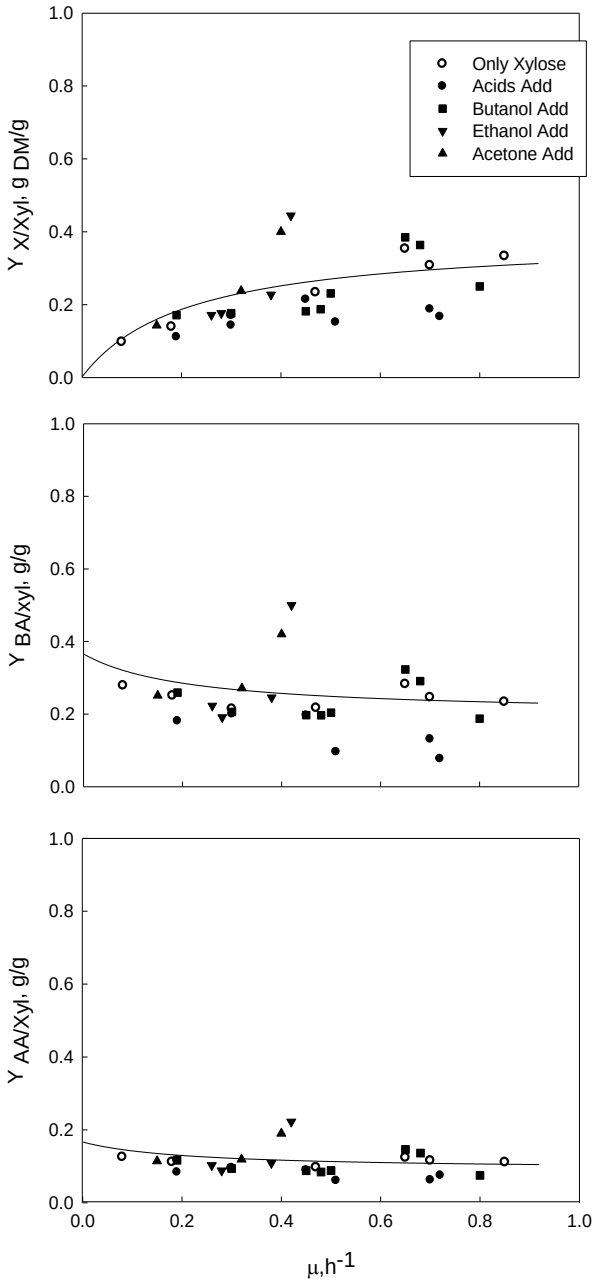

Figure 5. Fractional mass yield of cells and produced acids as a function of μ. Lines are plots of the theoretical values computed according to Eqs. (18)–(20)

Conclusions

The growth kinetics of *C. acetobutylicum* DSM 792 on xylose was investigated. Tests were carried out in a chemostat operated under controlled pH. The effects of ethanol, acetone and butanol on the growth rate of acidogenic cells were assessed. The experimental results were correlated by a multiple product-inhibited and interacted growth model. The kinetic model is relevant to design and to optimize a continuous bioreactor to produce ABE.

According to the maintenance model proposed by Pirt, a tool to assess the mass fractional yield of biomass and acids as a function of the specific growth rate was proposed. The agreement between the theoretical and experimental yields was fairly good.

5.2 Continuous xylose fermentation by *Clostridium acetobutylicum* – Assessment of solventogenic kinetics

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This paper reports results about specific butanol production rate of *Clostridium acetobutylicum* adopting xylose- a relevant fraction of lignocellulosic feedstocks for biofuel production- as carbon source. Tests were carried out in a CSTR equipped with a microfiltration unit. The dilution rate (D) ranged between 0.02 and 0.22 h^{-1} and the ratio R between the permeate stream rate and the stream fed at the reactor ranged between 14 and 88%. The biomass present in the broth was identify as heterogeneous cell population consisting of: acidogenic cells, solventogenic cells and spores. The results were processed to assess the concentration of acidogenic cells, solventogenic cells, spore. The specific butanol production rate was also assessed. The max butanol productivity was 1.3 g L^{-1} h^{-1} at D = 0.17 h^{-1} and R = 30%. A comparison between the results reported in a previous work carried out with lactose was made.

Introduction

The global warming, the stability of the fossil-source supply, and legislative restrictions regarding the use of non-renewable energy sources increased the interest in the biotechnology route for biofuel production (Bergthorsona et al., 2015). A potential alternative biofuel is the butanol. With respect to the ethanol, the well know biofuel, the butanol is characterized by: low heating value 25% larger than that of the ethanol; 6 time less evaporative; less hygroscopic; it may be transported in existing systems of gasoline. Butanol is 13.5 time less evaporative than gasoline and it may be mixed at high ratio with gasoline to fuel existing cars without modifications in gasoline-designed engine (Jin et al., 2011). Furthermore, as reported by Ujora et al. (2014), butanol can also be used: as a solvent (e.g. for paints, coatings, varnishes, resins, gums, dyes); in cosmetics (e.g. nail care products, shaving products, personal hygiene products); as a building block of chemicals (e.g. butyl acrylate, methacrylate). The Acetone–Butanol–Ethanol (ABE) fermentation is the main biotechnological route to produce butanol from renewable resources (Raganati et al., 2013; Ujora et al., 2014; Kheyrandish et al., 2015). However, the industrial development of the butanol production by the biotechnological route is still limited by several issues: i) the high cost of the substrate; ii) low productivity due to the end-product inhibition (Zeng et al., 1994); iii) the high product recovery cost (Napoli et al., 2012a; Wua et al., 2015). The cost of the substrate for the fermentation process represents about 60% of the overall production cost. Therefore, feedstocks available at high mass rate almost constant during the year and at low cost are a key issue for the success of the butanol production. Lignocellulosic materials, e.g., wood and agricultural residues, are potential feedstocks for ABE fermentation. Indeed, lignocellulosic biomass is: i) the most plentiful renewable resource of the planet; ii) it is made of potential fermentable sugars; iii) it is not useful as food resources; iv) it is

quite cheap (Friedl, 2012; Jang et al., 2012). Lignocellulosic materials consist mainly of cellulose and hemicellulose that may be hydrolyzed into pentoses and hexoses and these monomeric sugars may be metabolized by several Clostridium strains (Flickinger and Drew, 1999; Raganati et al., 2012). Several studies focused on xylose fermentation because it is main component of hemicelluloses and the fermentation is characterized by remarkable performances. Indeed, fermentations by Clostridia strains are characterized by yield of butanol from xylose very interesting. Shinto et al., 2008 reported the yield as high as 0.38 g/g for batch fermentations, remarkably larger than that assessed for glucose, 0.33 g/g. Zheng et al., 2013 operated a Clostridium saccharoperbutylacetonicum N1–4 continuous fermenter fed with xylose-supplemented medium and reported a butanol yield of 0.1 g/g.

Acetone-Butanol-Ethanol are produced during the last stage of the batch fermentation of different Clostridium strains (*Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium aurantibutyricum*). Batch fermentations are characterized by two phases, acidogenic phase and solventogenic phase. During the acidogenic phase the cells grow and produce acids (butyrate and acetate) and gas (carbon dioxide and hydrogen). The production of acids causes a decrease in pH and the environmental conditions become unfavourable to bacterial growth. Clostridia species respond to this new condition with a metabolic and morphological shift: the active cells become endospores, unable to grow and the acids are converted to solvents: Acetone–Butanol–Ethanol in a typical molar ratio 3:6:1. The fermentation process typically ends because high inhibiting solvent concentration (20-30 g L^{-1}) is approached (Jones and Woods, 1986). Reactor systems for the ABE fermentation include batch configuration as well as continuous configurations. The performance of the latter configurations is damaged by the low growth rate of clostridia cells under solventogenic conditions. Two potential solutions are the biofilm reactors (Napoli et al., 2010; Raganati et al., 2013; Liu et al., 2014) and the free-cell reactors coupled with recycling/confinement systems (Tashiro et al., 2005; Zheng et al., 2013). For all reactor configurations, a key issue for the design of the reactor and its optimization is the kinetics of the cell growth and butanol production.

This contribution reports the characterization of *Clostridium acetobutylicum* kinetics and butanol yields under solventogenic conditions adopting xylose as carbon source. The characterization has been carried out by means of a CSTR equipped with microfiltration unit used to confine the solventogenic cells in the reactor (Procentese et al., 2015). The dilution rate and the recycle ratio (the ratio between the volumetric flow rate through the microfiltration unit and the feed flow rate) ranged over wide intervals. Reactor performance were processed to assess the heterogeneous cell population (acidogenic, solventogenic, spore) in the reactor (Tracy et al., 2008) and the butanol production rate referred to the mass unit of solvetogenic cells. This paper integrates the previous model proposed for acidogenic cells reported for with xylose as carbon source (Procentese et al., 2014) with the aim to support the design and the optimization of continuous reactors.

Materials and methods

Microorganism and media

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. The stock cultures were reactivated according to the method suggested by the supplier and stored at -80°C. The cells were inoculated into 12 mL of synthetic pre-culture medium containing glucose (30 g L^{-1}) as carbon source. The carbon-less medium consisted of: 5g L^{-1} of yeast extract, 2 g L^{-1} of ammonium chloride (nitrogen source), 0.5g L^{-1}

KH_2PO_4- 0.5 g L^{-1} of K_2HPO_4 (buffer), 0.2 g L^{-1} of $MgSO_4.7H_2O$, 0.01 g L^{-1} of $MnSO_4.7H_2O$, 0.01 g L^{-1} of $FeSO_4.7H_2O$ (minerals). The medium was sterilized in autoclave (121 °C, 20min). The carbon source used during the continuous tests was xylose at 50 g L^{-1}. The chemicals and the yeast extract were from Sigma Aldrich.

Apparatus

The continuous fermentation tests were carried out in the apparatus sketched in Figure 1 consisting of a pH controller (Applikon Bio Controller ADI 1030), a fermenter and a microfiltration unit (Millipore Pellicon XL). The fermenter was a 1L jacketed vessel (Pyrex®) equipped with a gas sparger at the bottom. The gas stream was sterilized by filtration (cut-off 0.2 µm, Millipore). The culture was mixed by the re-circulation flow induced by the gas sparger at the bottom. A set of peristaltic pumps (Gilson Minipuls 3) was used to: i) deliver the liquid stream to the reactor (flow rate Q_0); ii) deliver the suspension to the microfiltration unit; iii) control the microfiltrate stream rate (flow rate Q_P). The liquid phase volume in the reactor was adjusted by changing the level of the overflow duct. The reactor vessel was sterilized in autoclave. The reactor temperature was controlled by means of a water stream coming from a thermostatic water bath and fed to the reactor jacket.

The microfiltration unit was equipped with three 0.22 micron-cartridges. One cartridge - 3.2 mL volume - was used for a 2-day filtration; the other two cartridges were washed to remove any biofouling formed during the filtration time. The membranes were washed for 24 h with a 0.01%w sodium dodecyl-sulphate (SDS) solution.

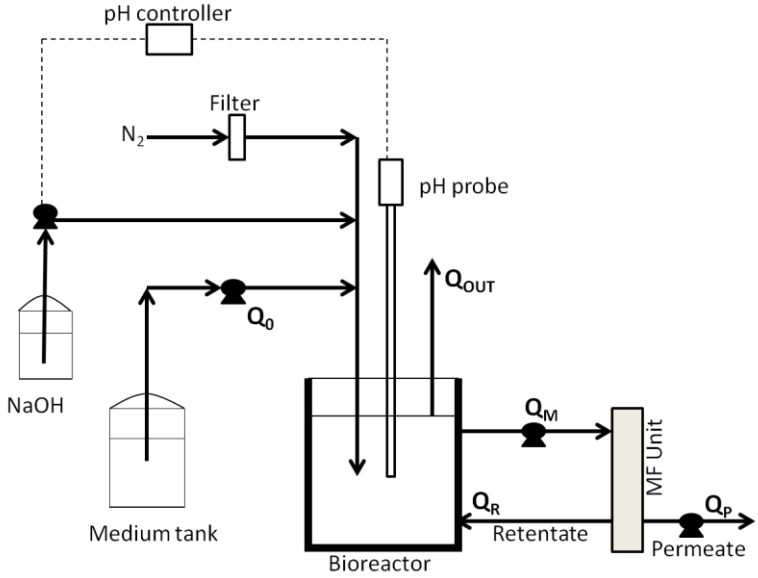

Figure 1. Outline of the apparatus adopted for continuous tests. Q_0 is the stream fed at the reactor, Q_P is the sterile broth (without cell), Q_{OUT} is the overflow duct containing cells.

Analytical methods

The cell density was measured as optical absorbance at 600 nm (OD600) using a spectrophotometer (Cary- 50 Varian). The calibration tests for the *C. acetobutylicum* dried mass indicated that 1 OD600/cm = 0.4 $g_{DM}L^{-1}$. The concentration of soluble species was measured in the liquid phase after centrifugation (10,000 rpm, 10min).

The pH was measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments).

The sugar concentration was measured by means of a high performance liquid chromatography (HPLC) (Agilent 1100 system). Deionized water was used as mobile phase at a 0.6 mLmin-1 flow rate. The sugars were separated on a 8 μm Hi-Plex H, 30 cm x 7.7 mm at room temperature and detected with a refractive index detector (RI).
The metabolite concentration (acids and solvents) was measured by means of a gas-chromatography (GC) apparatus (Agilent 7890A) equipped with a FID, and outfitted with a capillary column poraplot Q (25 m x 0.32 mm). Hexanoic acid was used as an internal standard.

Operating conditions and procedures

300 μL of stock culture were transferred into four 15-mL Hungate tubes containing the pre-culture medium (30 gL^{-1} of glucose). The pre-cultures were incubated for 2 days then 40 mL of active culture were inoculated in the reactor containing 0.5 L of test medium. After 6 h of batch culture the xylose-bearing stream was continuously fed to the reactor.
The stream fed to the reactor (flow rate Q_0) was split in two streams: Q_P) the sterile broth; Q_{OUT}) the broth stream spilled from the fermenter. The flow rate of the former stream was controlled by means of the peristaltic pump; the flow rate of the latter stream was measured at the overflow duct exit. Assuming that the density for the liquid streams was constant, the total mass balance and the mass balance for the cells extended to the reactor and the filtration unit were:

$$D = D_{OUT} + D_P \quad (1)$$

$$W = D_{OUT} \times X_T \quad (2)$$

where D is the dilution rate (Q_0/V), W_X the biomass productivity, X_T the total biomass concentration. D_{OUT} and D_P are the partial dilution rates assessed as

$$D_{OUT} = Q_{OUT} / V \quad (3)$$

$$D_P = Q_P / V \quad (4)$$

The recycle stream between the reactor and the microfiltration unit was characterized in terms of the recycle ratio (R) defined as (Meyer and Papoutsakis, 1989; Procentese et al., 2015):

$$R = \frac{Q_P}{Q_0} \quad (5)$$

The tests were carried out at 37°C. Nitrogen was sparged at the bottom of the reactor. The concentration of the xylose fed to the reactor was set at 50 g L^{-1}. D and R were changed between 0.02 and 0.22 h^{-1} and 14 and 88%, respectively. In particular, a set of runs was carried out setting R at 88% and changing D between 0.02 and 0.22 h^{-1}, a test campaign was carried out setting D at a pre-fixed value (0.07 and 0.1) and changing R between 14 and 88%.
The culture was periodically sampled to measure the biomass and metabolite concentrations until steady state conditions were reached. The data measured under steady-state conditions were averaged over a time interval of about 4–5 times the liquid space–time. The average concentration of xylose (C_{xyl}) and metabolites recorded during the tests were used to calculate the following data:

ξ_L overall lactose conversion, i.e. the ratio between the lactose converted and the initial lactose $(C_{L0}-C_L)/C_{L0}$;

$Y_{i/CL}$ lactose-to-"i-species" fractional yield coefficient, i.e., the ratio between the incremental "i-species" mass and the decrease of the substrate mass measured over the same time-interval;

Y_B/Y_{Sol} Butanol selectivity: the ratio between the butanol mass and the unit of mass of the acetone-butanol-ethanol.

Theoretical framework

The data measured in the fermenter under steady state conditions were processed to assess the kinetics of solventogenic cells and the specific butanol production rate.

The main assumptions of the model are:

i) the solventogenic cells are the only responsible for butanol production

ii) carbon source converts into CO_2 according to the Embden-Meyerhof-Parnas pathway (Jones and Woods, 1986)

iii) sterile feedings.

Biomass

The basic biomass pathway reported in Figure 2 was detailed in the previous work (Procentese et al., 2015) focused on solventogenic phase of *Clostridium acetobutylicum* adopting lactose as carbon source. The main assumptions of this model are:

An heterogeneous cell population is present in the reactor (acidogenic cells, solventogenic cells, spores);

The cell population may be modulated by acting on D and R;

The specific rate of cell lyses is negligible;

The specific rate of cell death is setting at μ_D = 0.0045 h^{-1}. Indeed, Flickinger and Drew (1999) reported that the cell death under a wide interval of adverse environmental conditions is characterized by a specific death rate ranging between 0.0015 and 0.009 h^{-1}. The medium value 0.0045 was selected and a sensitivity analysis is reported at the end of the "Results and Discussion" section.

The microbial population (acidogenic cells, solventogenic cells, spore) and the mass balance referred to each of the three classes of cells extended to the reactor and the filtration unit were reported in Procentese et al. (2015). In particular, it was:

$$D_{OUT}=\mu-\mu_s \quad (6)$$

where μ and μ_s are respectively, the acidogenic cell specific growth and the specific rate of solventogenetic cell formation.

The specific growth rate of acidogenic cells was estimated by means of the relationship proposed by Procentese et al. (2014) assessed for *C. acetobutylicum* fermentation on xylose:

$$\mu = \mu_{\max} \cdot \frac{C_{xyl}}{C_{xyl} + K_{Xyl}} \cdot \left(1-\frac{AA}{AA_{\max}}\right)^{n_{AA}} \cdot \left(1-\frac{BA}{BA_{\max}}\right)^{n_{BA}} \cdot \left(1-\frac{Ac}{Ac_{\max}}\right)^{n_{Ac}} \cdot \left(1-\frac{Et}{Et_{\max}}\right)^{n_{Et}} \cdot \left(1-\frac{B}{B_{\max}}\right)^{n_B} \quad (7)$$

Metabolites

The fermentation process of *C. acetobutylicum* on xylose during the acidogenic and solventogenic phases may be described by the reaction set reported in Table 1. The biomass in Eq. (8) of Table 1 is described as $C_4H_7O_2N$ according to the formula proposed by Zeng et al. (1996) for *C. butylicum*. Therefore, the biomass

is characterized by: molecular weight MWX=101 $gmol^{-1}$; carbon fraction σ=0.475; reductance degree γ=4.

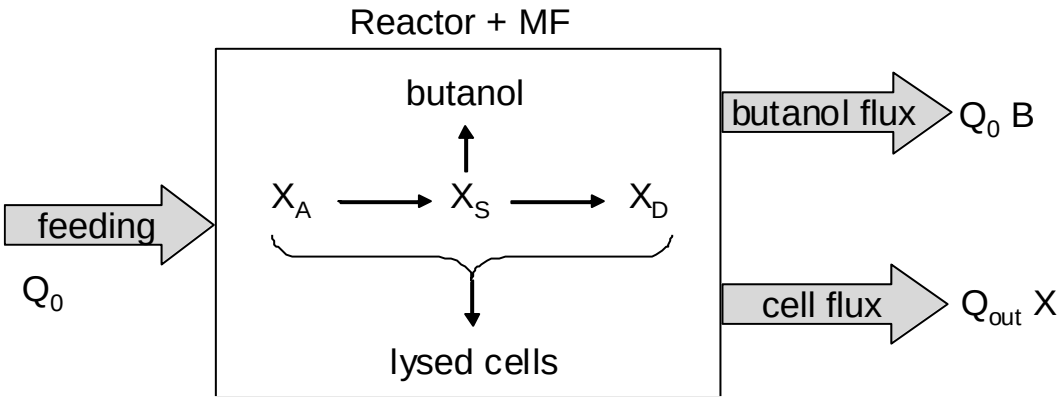

Figure 2. Synoptic scheme of the production/conversion processes investigated. In the Reactor + MF system there are three different kinds of cells. X_A acidogenic cells, X_S solventogenic cells and X_D spores. The total cells leave the system only with Q_{OUT}.

The molar balance referred to extracellular metabolites and extended to the reactor and the filtration unit reads (Procentese et al., 2015):

Acetic ac. $$F_{AA} = f_1^{AA} - f_3^{AA} - f_4^{AA} \quad (8)$$

Butyric ac. $$F_{BA} = f_2^{BA} - f_5^{BA} \quad (9)$$

Butanol $$F_B = f_4^B + f_5^B + f_6^B \quad (10)$$

Ethanol $$F_{Et} = f_7^{Et} + f_3^{Et} \quad (11)$$

Acetone $$F_{Ac} = f_3^{Ac} + f_4^{Ac} + f_5^{Ac} \quad (12)$$

where Fi is the molar net rate of production/uptake of the i-species measured during the tests, and f_n^i the molar rate of production/uptake of the i-species with respect to the reaction "n" of Table 1. In particular, "i" may be: Ac - acetone, AA – acetic acid, B – butanol, BA – butyric acid, Et – ethanol. According to the metabolic reactions reported in Table 1, the following stoichiometric relationships were used:

$$f_4^{Ac} = f_4^{AA} \quad (13)$$

$$f_5^{Ac} = f_5^{BA} \quad (14)$$

$$f_4^B = \frac{f_4^{AA}}{2} \quad (15)$$

$$f_3^{Et} = f_3^{AA} \quad (16)$$

A set of six independent equations in the six variables $\left(f_1^{AA}, f_4^{AA}, f_2^{BA}, f_5^{BA}, f_6^B, f_7^{Et}\right)$ is obtained (Procentese et al., 2015).

The production flux of acetic acid $\left(f_1^{AA}\right)$ and of butyric acid $\left(f_2^{BA}\right)$ are used to assess the acidogenic cell concentration according to the relationship proposed by Napoli et al. (2012b):

$$X_A = \frac{Y_{ATP}\left(2 f_1^{AA} + 3 f_2^{BA}\right)}{\mu} \quad (17)$$

where Y_{ATP} is the ATP yield - expressed as dry mass of cells produced per mole of ATP generated- proposed by Bauchop and Elsden (1960).

The values of $f_1^{AA}, f_4^{AA}, f_2^{BA}, f_5^{BA}, f_6^{B}, f_7^{Et}$, X_A, were used to assess X_S, X_D and μs by resolving the set of Eq.s (8) trough (12).

The production rate of solvents referred to the mass unit of solventogenic cells (rs) was assessed according to Eq. (18):

$$r_S = \frac{DC_S}{X_S} \tag{18}$$

Where r_S is the production rate of the solvent (A-acetone, B-butanol, E-ethanol) and C_S the solvent concentration. As regard the butanol production rate, the data of r_B were interpreted taking into account that xylose, acetic acid and butyric acid are the substrates and butanol is the inhibition product. In particular, the Monod-Boulton model proposed for processes inhibited by products (Arellano et al., 2007) was used:

$$r_B = r_{B,MAX}\left(\frac{C_{xyl}}{K_{\mathbf{xyl},B} + C_{xyl}}\right)\left(\frac{AA}{K_{AA} + AA}\right)\left(\frac{BA}{K_{BA} + BA}\right)\left(\frac{K_B}{K_B + B}\right) \tag{19}$$

where r_{BMAX} is the maximum specific production rate, $K_{xyl,B}$, K_{AA}, K_{BA} and K_B are the constants of the model.

Computation procedure

The value of $r_{B,MAX}$, $K_{xyl,B}$, K_{AA}, K_{BA} and K_B were determined by a parametric inference procedure. The data of r_B measured during the continuous tests were processed by means of a regression tool of Sigma Plot®.

Results and discussion

Solvent production

Figure 3A-B reports metabolites (acids and solvents), the concentration of xylose (C_{xyl}) and cells concentration (X_{TOT}), measured during steady states of the fermentation, as a function of the dilution rate (D) at recycle ratio (R) set at 30%. The pH was set at 4.7 in all operating conditions adopted. The xylose conversion and the concentration of products (cells and metabolites) decrease with the dilution rate. Acetic acid, butyric acid, acetone and ethanol approached a constant value as D was larger than 0.12 h^{-1}. Butanol concentration decreases remarkably with D and in particular when larger than 0.17 h^{-1} the butanol concentration decreases remarkably. The xylose conversion (Fig. 3B) decreases with D and it is expected to be complete at D≈0.018 h^{-1}. Figure 3C reports the specific productivity of butanol and of solvents assessed for the tests reported in Figure 3A-B. Both productivities are characterized by a maximum at D = 0.17h^{-1}. The decrease of the solvent productivity at D larger than 0.17 h^{-1} is due to the remarkable decrease of the butanol concentration with D.

The comparison with the results obtained from tests carried out with a lactose bearing medium is particularly interesting. The xylose may be converted completely setting a finite D while the lactose conversion was always less than 70% for each the investigated D (Procentese et al., 2015). As regards the biomass concentration it is worth to note that at R=30% it was three time larger than that measured during tests carried out with lactose as carbon source and at R=88% (Procentese et al., 2015).

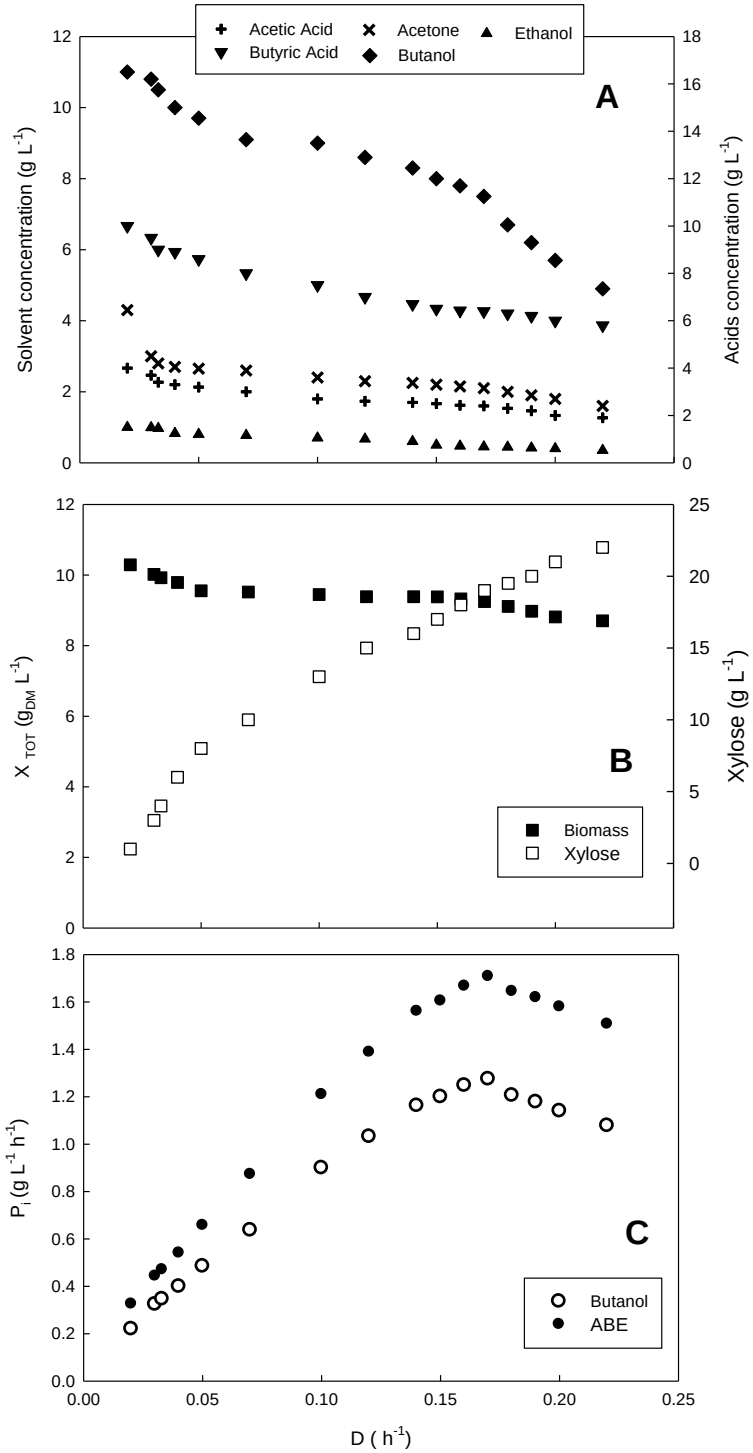

Figure 3. Data referred to steady states of continuous fermentation tests as a function of the dilution rate. R = 30%. A) Concentration of metabolites B) Concentration of xylose and biomass C) Solvent productivity.

Table 2 reports: xylose conversion degree (ξ_{xyl}), butanol yield (Y_B/C_{xyl}) and selectivity (Y_B /Y_{Sol}) for different D at R = 30%. As expected, increasing D up to 0.17 h^{-1} the xylose conversion degree decrease, the butanol yield increased till a constant value of about 0.24 g g^{-1}, the butanol selectivity increased till a constant value of about 0.74 g g^{-1}. Both butanol yield and butanol selectivity decrease at D ≥0.18h^{-1} .

D (h^{-1})	ξxyl	$Y_{B/C_{xyl}}$	Selectivity ($Y_{B/C_{xyl}}/Y_{Sol/C_{xyl}}$)
0.02	0.98	0.22	0.67
0.03	0.94	0.23	0.73
0.033	0.92	0.24	0.74
0.04	0.88	0.23	0.74
0.05	0.84	0.23	0.74
0.07	0.80	0.23	0.74
0.1	0.74	0.24	0.74
0.12	0.70	0.25	0.74
0.15	0.66	0.24	0.75
0.17	0.62	0.24	0.75

Table 2. Xylose conversion degree (ξ_{xyl}), butanol yield (Y_B/C_{xyl}) and selectivity ($Y_B/C_{xyl}/Y_{Sol}/C_{xyl}$) of the continuous fermentation in the reactor-MF plant: effects of D at R 30%. Xylose concentration in the feeding 50 g L^{-1}.

Table 3 reports the results of the two set of tests carried at a pre-set D (0.07 and 0.1 h^{-1}) and changing R. The butanol and the total biomass concentration measured increase with R, for all values of D set. Furthermore, the specific productivity of both butanol and solvents calculated increase with R for all values of D set. The maximum productivity of both butanol and ABE was found at D = 0.1 h^{-1} and R = 88%: 1.10 and 1.55 g L^{-1} h^{-1}, respectively.

These trends are in good agreement with those reported by previous work on lactose (Procentese et al., 2015). Indeed, with the same apparatus and operated conditions the productivity of both butanol and ABE increased with R for all values of D. As expected, in the present paper the total cells concentration and the maximum productivity of both butanol and solvent were higher than values reported with lactose. Indeed, as reported by Yu et al. (2007) monomeric sugars are preferred carbon source for *Clostridium* with respect to lactose.

Present results are now compared with Zheng et al. (2013) that used *C. saccharoperbutylacetonicum* N1-4 and xylose as carbon source. Their apparatus was similar to that adopted in the present study even though the operating conditions were different: R=100% and D ranged between 0.14 to 0.85 h^{-1}. However, the behaviour of the fermentation performance reported in the present investigation agree with those reported by Zheng et al. (2013): the concentration of butanol decreases and the productivity of butanol increases with D.

Cell related kinetics

The concentration of acidogenic cells, solventogenic cells and spores were assessed according to the mechanistic framework previously described which was adapted and improved from the previous work (Procentese et al., 2015). Figure 4A reports the total cell concentration measured during the continuous fermentation tests as well as the calculated concentration of acidogenic cells, solventogenic cells and spores for continuous fermentation tests characterized by R=30% and D ranging between 0.02 and 0.22 h^{-1}. The concentration of acidogenic cells increases linearly with D while the spore concentration decreases exponentially with D. The concentration of solventogenic cells increases of about 10% at low D, decreases at D > 0.04 h^{-1} and it is constant at D>0.16h^{-1}. Figure 4B reports the specific rate of acidogenic cell growth (μ) and of solventogenic cell production (μ_S) assessed according to the relationship proposed by Eq. (7) and Eq. (6), respectively.

Operating conditions		Measured data				Calculated data						
D (h^{-1})	R %	Xyl (gL^{-1})	B (gL^{-1})	B/ABE (-)	X_{TOT} ($g_{DM}L^{-1}$)	P_{But} ($gL^{-1}h^{-1}$)	P_{ABE} ($gL^{-1}h^{-1}$)	X_A ($g_{DM}L^{-1}$)	X_S ($g_{DM}L^{-1}$)	X_D ($g_{DM}L^{-1}$)	μ (h^{-1})	μ_S (h^{-1})
0.1	14	17	7.8	0.76	8.30	0.78	1.0	1.86	6.15	0.30	0.40	0.31
0.1	30	13	9	0.74	9.40	0.90	1.21	2.47	6.61	0.35	0.31	0.23
0.1	54	8.5	9.10	0.73	11.8	0.91	1.24	2.83	8.44	0.57	0.27	0.21
0.1	88	8	11	0.71	14.1	1.10	1.55	4.57	8.72	0.83	0.14	0.09
0.07	14	15	8.1	0.74	8.40	0.56	0.76	1.41	6.57	0.46	0.38	0.32
0.07	30	10	9.1	0.73	9.50	0.63	0.87	1.98	6.98	0.54	0.27	0.22
0.07	54	7	9.7	0.71	12	0.67	0.92	2.35	8.76	0.91	0.22	0.17
0.07	88	6	11.3	0.67	14.50	0.79	1.17	4.04	8.83	1.63	0.08	0.06

Table 3. Main data of the continuous fermentation in the reactor-MF plant: effects of R at two values of D. Xylose concentration in the feeding 50 g L^{-1}.

At D between 0.02 and 0.12 h^{-1}, both μ_S and μ increase. Figure 4B shows that μ is larger than D_{OUT} for all investigated D. However, acidogenic cell concentration did not increase in the reactor as a result of the solventogenic cell production path. Indeed, the difference between μ and μ_S is just D_{OUT} as reported in Eq. (6).

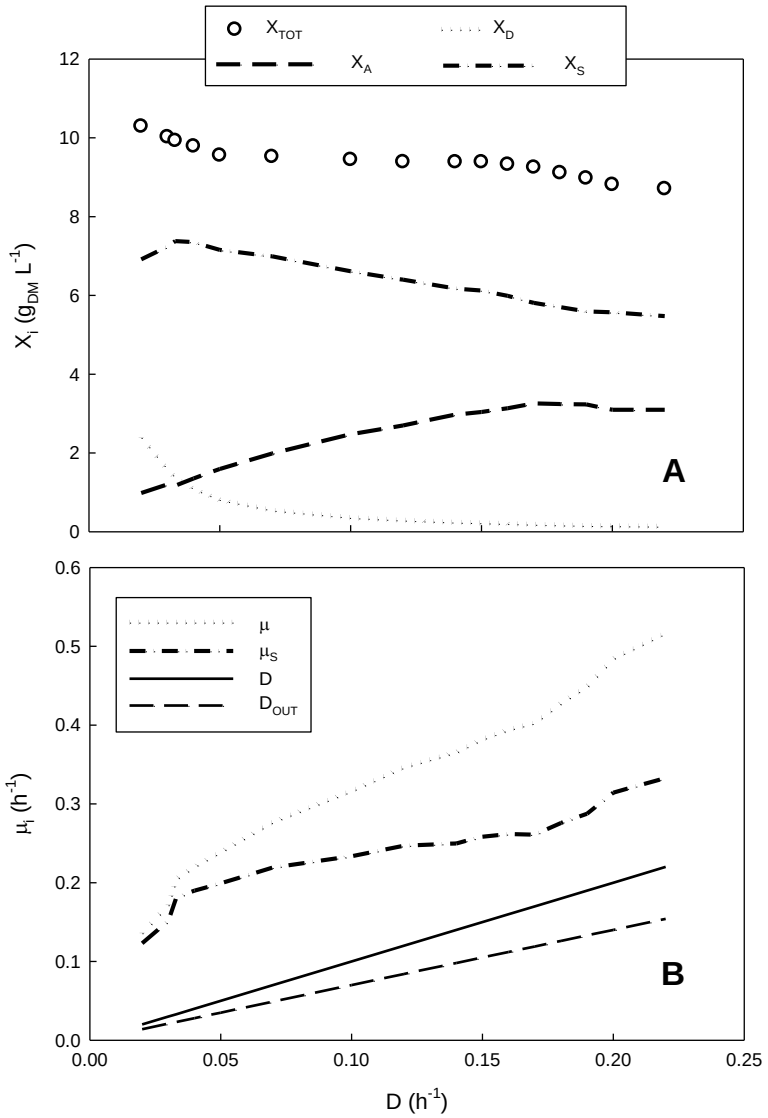

Figure 4. Data measured (points) and calculated (lines) for steady states of continuous fermentations as a function of the dilution rate. R = 30%. A) Concentration of acidogenic cells (X_A), solventogenic cells (X_S) and spores (X_D). B) Specific growth rate: μ (Eq.7) and μ_S (Eq.6).

Figure 5A reports D_{OUT} measured (Eq. 2) vs. D_{OUT} assessed from the model (Eq. 6). Inspection of the parity plot indicates that the departure of model predictions from experimental data points is fairly small, typically within 20%.

Kinetics of butanol production

The production rate of butanol referred to the mass unit of solventogenic cells (r_B) – Eq. (18) - was calculated for all tests. Data regression according to Eq. (19) provided the assessment of the relationship constants. The results of the best-fit procedure are reported in Table 4. It is interesting to compare the constant values obtained in this work with those reported in the previous work on lactose (Procentese et al., 2015). On lactose r_{BMAX} was assessed at 4.7g_B g_{DM}^{-1} h^{-1}, in the present work a

value of 6.3 g_B g_{DM}^{-1} h^{-1} was achieved. Furthermore, K_{AA}, K_{BA}, and K_B values are similar to those reported on lactose as carbon source. This result suggests that the acids up-take and butanol inhibition don't depend on carbon source. $K_{xyl,B}$ = 15 g L^{-1} suggested that the butanol production rate depends on the xylose. As a consequence, the conversion time of all xylose to butanol depends on the xylose concentration. On the contrary, K_L was about ≈ 0 g L^{-1} this suggested that the butanol production rate does not depend on the lactose concentration. As a consequence, the conversion time of all lactose to butanol does not depend on the lactose concentration: reaction rate of zero order. Furthermore, it is interesting to compare the $K_{xyl,B}$ value obtained in this work with the xylose concentration in a typical lignocellulosic biomass. As reported by Ezejj et al. (2007), glucose: mannose: arabinose: xylose in a typical lignocellulosic biomass are present in mass ratio 5:1:2:4. If a initial total sugar concentration of about 60g L^{-1} is taken into account, the initial xylose concentration would be 20 g L^{-1} which is comparable to $K_{xyl,B}$. For these reasons the conversion rate of all xylose to butanol, when a hydrolisate of lignocellulose biomass is used as substrate for the fermentation, would linearly depend on xylose concentration as xylose concentration decreases.

Substrate	$r_{B,MAX}$ (g_B $g_{DM}^{-1}h^{-1}$)	K ($g\ L^{-1}$)	K_{AA} ($g\ L^{-1}$)	K_{BA} ($g\ L^{-1}$)	K_B ($g\ L^{-1}$)	Reference
Lactose	4.7	≈ 0	0.8	0.25	0.48	Procentese et al., 2015
Xylose	6.3	15	0.7	0.18	0.51	This study

Table 4. Results of the best-fit procedure of eq.(19) and comparison with results reported on lactose (Procentese et al., 2015)

Figure 5B reports the parity plots of the measured r_B with the value assessed according to Eq. (19) and using model parameters reported in Table4. The error between the model prediction and experimental data is typically within 20%. Therefore, the soundness of the proposed theoretical framework may be claimed. Figure 6 reports the specific solvent production rates assessed according to (Eq.18) as a function of the dilution rate at R set at 30%. The specific production rate of butanol is maximum at D 0.17 h^{-1} , that of the ethanol and of acetone increases with D. The different behaviour of the specific production rate at the largest D may be interpreted taking into account the value of the butanol concentration measured under these conditions as well as the cells concentration. Indeed, the butanol concentration (8g L^{-1}) is close to the maximum inhibiting threshold and the solventogenic cells concentration is lower than those reported at D≤ 0.17h^{-1}. Instead, ethanol and acetone concentration are still lower than the inhibiting threshold. For this reason, it is expected that the solventogenic cells adapt themselves favouring the production of solvents as ethanol and acetone which are far from the inhibiting threshold concentration. This behaviour is founded in the decrease of the butanol selectivity reported at D≥0.18 h^{-1}. The observed decrease of the butanol production rate at high D was not observed during tests carried out using lactose as carbon source (Procentese et al., 2015). The previous paper also reported the decrease of solventogenic cells with the D but the butanol concentration was about 4 g L^{-1} and this value was still far from inhibiting butanol concentration.

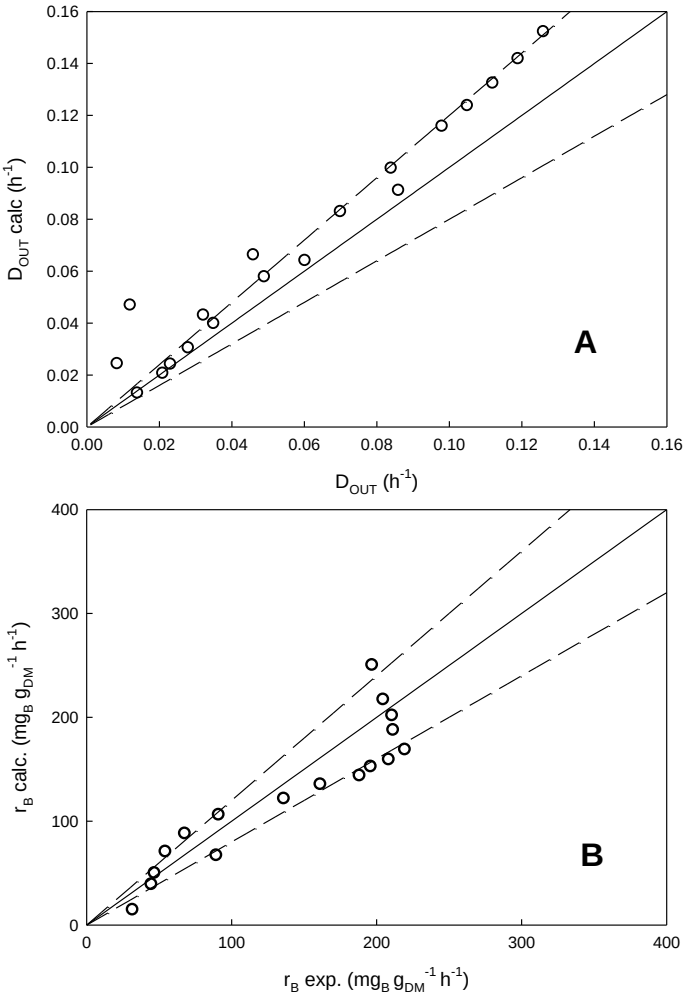

Figure 5. A)D_{OUT} calculated vs. D_{OUT} measured. B)r_B calculated (Eq.19) vs. measured (Eq.18). Dashed lines : ±20% error.

Sensitivity analysis of the specific rate of spore formation

The results reported in the previous sections have been obtained setting the specific rate of spore formation (μ_D) at 0.0045h^{-1}.
According to Flickinger and Drew (1999) μ_D ranges between 0.0015 and 0.009h^{-1}: the value of μ_D may range over an order of magnitude. Results of the model have been assessed setting μ_D at 0.0015 and at 0.009 h^{-1}. The values of the kinetic model constants don't change with μ_D.

Conclusions

The characterization of *C. acetobutylicum* DSM 792 on xylose during solventogenic phase was carried out in a CSTR equipped with a microfiltration unit. The biomass present in the broth was identified as: acidogenic cells, solventogenic cells and spores. The butanol production rate was referred only to the mass unit of solventogenic cells. A comparison between the results reported in a previous work carried out with lactose was made. The model obtained combining the kinetic and stoichiometry in acidogenesis (Procentese et al., 2014) and solventogenesis (this work) may be adopted for the design and the optimization of reactor systems characterized by a the heterogeneous cell population.

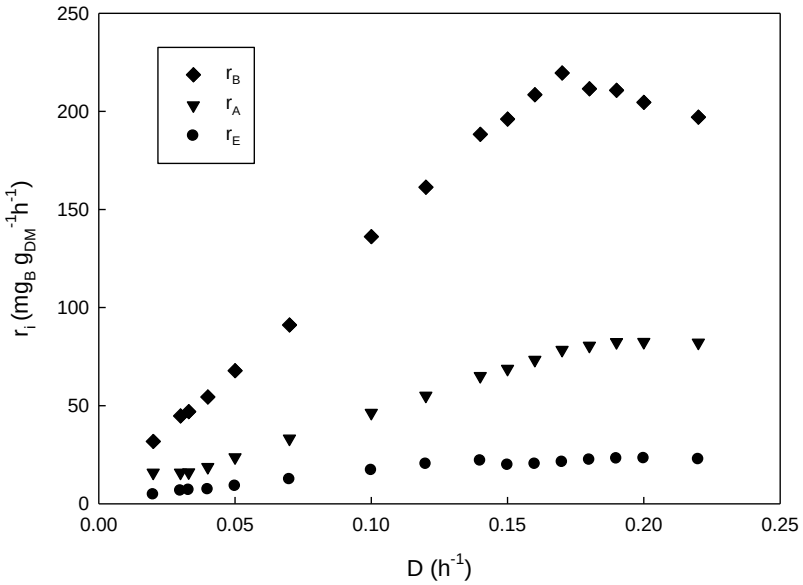

Figure 6. Specific solvent production rates. r_B, r_A and r_E measured (Eq.18) as function of the dilution rate at R set at 30%.

Acknowledgment

The authors thank the Ministero dello Sviluppo Economico for their financial support at the project EuroTransBio ETB-2012-16 OPTISOLV (Development, optimization and scale-up of biological solvent production).

5.3 Continuous lactose fermentation by Clostridium acetobutylicum – Assessment of solventogenic kinetics

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This work reports results about specific butanol production rate by continuous cultures of *Clostridium acetobutylicum*. The tests were carried out adopting lactose as carbon source to mime cheese-whey. A continuous stirred tank reactor equipped with a microfiltration unit was adopted. The dilution rate (D) ranged between 0.02 and 0.15 h^{-1} and the ratio R between the permeate stream rate and the stream fed at the reactor ranged between 14 and 95%. For each set of D and R values, the continuous cultures were characterized in terms of concentration of cells, acids and solvents. The results were processed to assess the concentration of acidogenic cells, solventogenic cells, spore and the specific butanol production rate. The max butanol productivity was 0.5 g L^{-1} h^{-1} at D = 0.1 h^{-1} and R = 95%. The butanol production rate

referred to solventogenic cells was expressed as a function of the concentration of lactose, acids and butanol.

Introduction

The worldwide demand of fuels as well as of chemical building blocks is continuously increasing and conflicts with the known depletion of fossil-fuel sources and the awareness of the environmental damages produced by fossil-derived product/processes. A potential solution to these two issues is the adoption of alternative routes to the petrochemical productions. Butanol is an alternative fuel, it may be produced by alternative routes as thermochemical and biotechnological ones. The butanol can also be used (Cascone, 2008; Lee et al., 2008; Jin et al., 2011): as a solvent (e.g. for paints, coatings, varnishes, resins, gums, dyes); in cosmetics (e.g. nail care products, eye makeup, lipsticks, shaving products, personal hygiene products); as a building block of chemicals (e.g. butyl acrylate, methacrylate). Main differences with respect to the ethanol – a well known biofuel – are (Durre, 2007; Lee et al., 2008a): the low heating value is 25% lager; 6 time less evaporative; less hygroscopic; it may be transported in existing systems of gasoline. Butanol is 13.5 time less evaporative than gasoline and may be mixed at high ratios with gasoline to fuel existing cars without modifications in gasoline-designed engine. Several enterprises claim that butanol can be used as a total replacement fuel for gasoline without any modifications to car engines. Indeed, the butanol beat has been accept by several enterprise/company. Among them it is possible to list small and large companies in Europe (e.g. Sovert, ButalcoGmBH), in the USA (e.g. Environmental Energy Inc., GEVO^{TM}, Butamax^{TM}, Cobalt Technologies) and in Asia (e.g. Cathay Industrial Biotech). The biotechnological routes to produce butanol include the Acetone–Butanol–Ethanol (ABE) fermentation. It was proposed by Weizmann et al. (1937) and was one of the most widely used industrial fermentation processes in the first half of the 20th century. The advent of the petrochemical industry at the begin of the second half of the last century made the production of acetone and butanol by fermentation uncompetitive and plants were closed. Nowadays, the progress of the research have revived the interest in the fermentative route (Ezeji et al., 2007a; Raganati et al., 2013; Pinto Mariano et al., 2013). However, the industrial development of the butanol production by the biotechnological route is still limited by several issues that include: the high cost of the substrate; the low product concentration and productivity in fermentation due to end-product inhibition (Jones and Woods, 1986; Zeng et al., 1994); the high product recovery cost (Ezeji et al., 2005; Napoli et al., 2012a;). The cost of the substrate for the fermentation process represents about 60% of the overall production cost. Therefore, feedstocks available at high mass rate almost constant during the year and at low cost are a key issue for the success of the butanol production.

Cheese-whey is a wastewater stream released from the cheese industry. It is produced at almost constant rate during the year at quite high mass rate. A small dairy produces more than 20 $m^3 day^{-1}$ of wastewater, a polluted stream comparable to that released by a community of about 10.000 people. The Biochemical Oxygen Demand (BOD) is very high – typically larger than 2000 mg L^{-1} - and the COD is about 50-70 g L^{-1} (Najafpour et al., 2008). The lactose is typically present at concentration larger than 30-50 g L^{-1} and is the main responsible of the polluting behaviour of the cheese-whey. As a high polluted stream, cheese-whey must be remediated before to be delivered at a sewer system. The scenario becomes even

worst in Italy where there are about 2.800 dairies that produce between 8 and 10 million $m^3 year^{-1}$ of cheese-whey.
ABE is typically produced during the last stage of the batch fermentation of some Clostridium strains (*C. acetobutylicum, C. beijerinckii, C. aurantibutyricum*). *C. acetobutylicum* batch fermentation is characterized by two phases: acidogenic and solventogenic phases. During the acidogenic phase the cells grow and produce: acids (butyric and acetic acid), carbon dioxide, and hydrogen. The production of acids causes a decrease in pH, the *C. acetobutylicum* cells adapt themselves to the acid environment by a metabolic and a morphological shift. The solventogenesis phase establishes and it is characterized by: i) the end of the exponential growth phase; ii) the active cells become endospores, unable to grow; iii) the acids are converted to solvents, Acetone-Butanol-Ethanol (typical molar ratio 3:6:1). The fermentation process typically ends because high inhibiting solvent concentration (20-30 g L^{-1}) is approached: The harsh conditions induce cell sporulation and/or cell lysis (Jones and Woods, 1986; Tracy et al., 2008). During the solventogenesis phase the cell growth rate is quite low. Therefore, under continuous process the reactor configuration must be selected to prevent reactor washout (Huang et al., 2004; Napoli et al, 2010; Raganati et al., 2013). The selection and the optimization of the continuous reactor require the knowledge of the kinetics – cell growth rate, butanol production , etc. - of the process as a function of the operating conditions and of the species involved in the fermentation.. The kinetics under solventogenic conditions assumes a key role to optimise the butanol production. Meyer and Papoutsakis (1989) reported a study on the solventogenisis phase adopting a continuous stirred tank reactor equipped with a microfiltration unit to confine the solventogenic cells in the reactor. They investigated the metabolites production at different dilution rate (D = volumetric flow rate / reaction volume) and different recycle ratio (R – ratio between the volumetric flow rate permeated across the microfiltration unit and the feed flow rate). Authors focused only on solventogenic cells even though a population of cells may be found in a continuous reactor (Zheng et al., 2013). Indeed, Napoli et al. (2011, 2012b) and Zheng et al. (2013) pointed out the simultaneous presence of acidogenic cells and solventogenic cells in the fermenter.
This contribution reports the characterization of *Clostridium acetobutylicum* kinetics under solventogenic conditions. This contribution integrates the model proposed by Napoli et al. (2009) for acidogenic cells. The acidogenic cells model and the solventogenic cells model are finalized to support the design of continuous reactors. A continuous stirred tank reactor equipped with microfiltration unit was used to confine the solventogenic cells in the reactor. The dilution rate and the recycle ratio (the ratio between the volumetric flow rate through the microfiltration unit and the feed flow rate) ranged over wide intervals. Lactose was adopted as carbon source as mime of the cheese-whey. The fermentation tests were aimed: i) to assess the butanol production rate as a function of the operating conditions; ii) to assess the kinetics under solventogenic phases.

Materials and methods

Microorganism and media

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Stock cultures were reactivated according to the method suggested by the supplier and stored at -80°C. The cells were inoculated into 12 mL synthetic pre-culture medium containing glucose (30 g L^{-1}) as carbon source. The carbon-less medium consisted of: 5 g L^{-1} Yeast Extract, 2 g L^{-1} ammonium chloride (nitrogen source), 0.5 g L^{-1} KH_2PO_4- 0.5 g

L^{-1} K_2HPO_4 (buffer), 0.2 g L^{-1} $MgSO_4.7H_2O$, 0.01 g L^{-1} $MnSO_4.7H_2O$, 0.01 g L^{-1} $FeSO_4.7H_2O$ (minerals). The medium was sterilized in autoclave (121 °C, 20min). The carbon source adopted during the continuous tests was lactose at 50 g L^{-1}. Chemicals and yeast extract were from Sigma Aldrich.

Apparatus

The continuous fermentation tests were carried out in the apparatus sketched in Fig. 1. It consisted of a fermenter, a pH controller (Applikon Bio Controller ADI 1030) and a microfiltration unit (Millipore Pellicon XL). The fermenter was 1 L jacketed vessel (Pyrex®) equipped with a gas sparger at the bottom. The gas stream was sterilized by filtration (cut-off 0.2 µm, Millipore). The culture mixing was provided by the re-circulation flow induced by gas bubbling. A set of peristaltic pumps (Gilson Minipuls3) were adopted to: i) deliver the liquid stream at the reactor (flow rate Q_0); ii) deliver the suspension at the microfiltration unit; iii) control the microfiltrate stream rate (flow rate Q_P). The liquid phase volume in the reactor was tuned by changing the level of the overflow duct. The reactor vessel was sterilized in autoclave.
Reactor temperature was controlled by means of a water stream delivered by a thermostatic water bath and fed at the reactor jacket.
The microfiltration unit was equipped with 0.22 micron cartridges. One cartridge-3.2mL volume - was adopted for 1 day filtration, the other two cartridges were washed to remove possible biofouling formed during the filtration time. Membranes were washed for 24 h with a 0.01%w sodium dodecyl sulphate (SDS) solution.

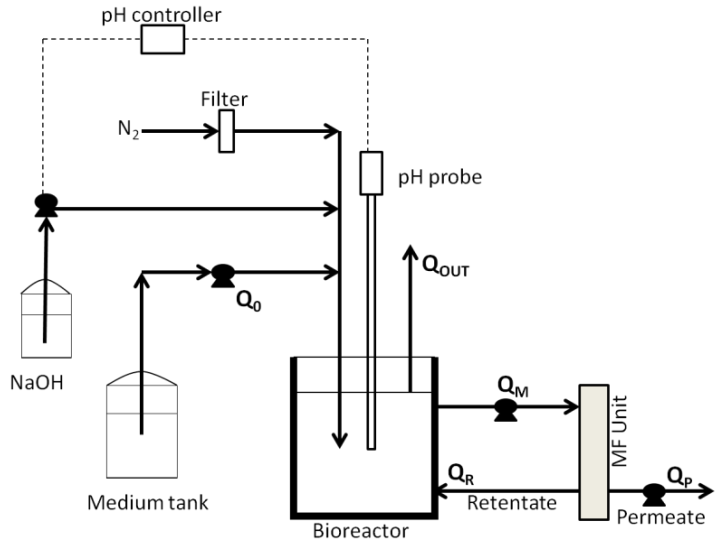

Figure 1. Outline of the apparatus adopted for continuous tests. Q_0 is the stream fed at the reactor, Q_P is the sterile broth (without cell), Q_{OUT} is the overflow duct containing cells. The liquid phase volume in the reactor was tuned by changing the level of the overflow duct. The N_2 was sparged in the same line of the stream fed in order to hold the line clean.

Analytical methods

pH was measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments). Cell density was measured as optical absorbance at 600 nm (OD 600) using a spectrophotometer (Cary- 50 Varian). Calibration tests for C. acetobutylicum dried mass indicated that 1 OD600/cm = 0.4 $g_{DM}L^{-1}$. The concentration of soluble species was measured in the liquid phase after centrifugation (10,000 rpm, 10 min).

Sugar concentration was measured by means of a high performance liquid chromatography (HPLC) (Agilent 1100 system). Deionized water was used as mobile phase at 0.6 mL min^{-1} flow rate. The sugars were separated on a 8 µm Hi-Plex H, 30 cm x 7.7 mm at room temperature and detected with a refractive index detector (RI).
Metabolite concentration (acids and solvents) was measured by means of a gas-chromatography (GC) apparatus (Agilent 7890A) equipped with a FID, and outfitted with a capillary column poraplot Q (25 m x 0.32 mm). Hexanoic acid was adopted as an internal standard.

Operating conditions and procedures

300 µL of stock culture were transferred into four 15-mL Hungate tubes containing the pre-culture medium (30 g L^{-1} of glucose). The pre-cultures were incubated for 2 days, then 40 mL of active culture were inoculated in the reactor containing 0.5 L of test medium. After 6 h of batch culture the lactose-bearing stream was continuously fed to the reactor.

The stream fed at the reactor (flow rate Q_0) was split in two streams: Q_P) the sterile broth; Q_{OUT}) the broth stream spilled from the fermenter. The flow rate of the first stream was controlled by means of the peristaltic pump; the flow rate of the latter stream was measured at the overflow duct exit. Assuming constant density for the liquid streams, the total mass balance and the mass balance for the cells were:

$$D = D_{OUT} + D_P \quad (1)$$

$$W = D_{OUT} \times X_T \quad (2)$$

where D is the dilution rate (Q_0/V), W_X the biomass productivity, X_T the total biomass concentration, D_{OUT} and D_P are the partial dilution rate assessed as

$$D_{OUT} = Q_{OUT}/V \quad (3)$$

$$D_P = Q_P/V \quad (4)$$

According to Meyer et al. (1989), the recycle stream between the reactor and the microfiltration unit was characterized in terms of the recycle ratio (R) defined as:

$$R = \frac{Q_P}{Q_0} \quad (5)$$

Tests were carried out at 37°C. Nitrogen was sparged at the bottom of the reactor. The lactose concentration in the reactor feeding was set at 50 g L^{-1}. D and R were changed between 0.02 and 0.15 h^{-1} and 14 and 95%, respectively. In particular, a set of runs was carried out setting R at 88% and changing D between 0.02 and 0.15 h^{-1}, a test campaign was carried out setting D at a pre-fixed value (0.07 and 0.1) and changing R between 14 and 95%.
The culture was periodically sampled to measure biomass and metabolite concentrations until steady-state conditions were reached. Data measured under steady-state conditions were averaged over a time interval of about 4–5 times the liquid space–time (=1/D). Average data of concentration of lactose (C_L) and metabolites recorded during the tests were used to calculate the following data:

ξ_L overall lactose conversion, i.e. the ratio between the lactose converted and the initial lactose $(C_{L0}-C_L)/C_{L0}$;

$Y_{i/CL}$ lactose-to-“i-species” fractional yield coefficient, i.e., the ratio between the incremental “i-species” mass and the decrease of the substrate mass measured over the same time-interval;

Y_B/Y_{Sol} Butanol selectivity: the ratio between the butanol mass and the unit of mass of the acetone-butanol-ethanol.

Theoretical framework

The data measured in the fermenter under steady state conditions were processed to assess kinetics regarding solventogenic cells and the specific butanol production rate. Figure 2 reports a synoptic scheme of the basic idea: the solventogenic cells are the only responsible of the butanol production and the cell population may be modulated by acting on D and R. Main assumptions of the model are: i) three types of cells are present in the cultures (acidogenic cells, solventogenic cells, spores); ii) carbon source converts into CO_2 according to the Embden-Meyerhof-Parnas pathway (Jones and Woods, 1986); iii) sterile feedings.

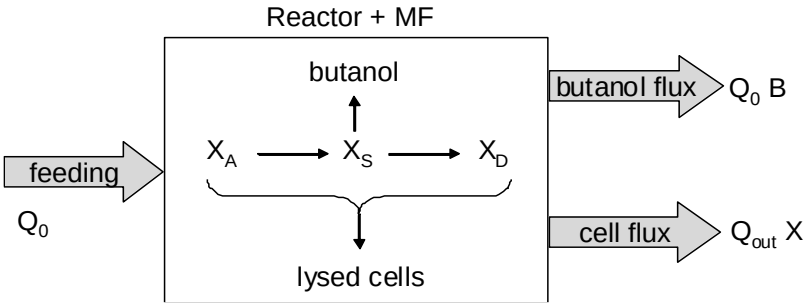

Figure 2. Synoptic scheme of the production/conversion processes investigated. Three kinds of cells are present in the Reactor + MF system: X_A - acidogenic cells; X_S - solventogenic cells; X_D - spores. The cells flow out the system by mans of Q_{OUT}.

Biomass

Sarrafzadeh et al. (2005) propose to model the transformation paths of the microbial population under solventogenesis conditions by the following set of reactions:

$$L \xrightarrow{\mu} X_A \quad (6)$$

$$X_A \xrightarrow{\mu_S} X_S \quad \text{(solventogenic cells formation)} \quad (7)$$

$$X_S \xrightarrow{\mu_D} X_D \quad \text{(spore formation)} \quad (8)$$

$$X_A, X_S, X_D \xrightarrow{\mu_{Lysis}} cell\ lysis \quad (9)$$

where X_A, X_S, X_D are the acidogenic cells, solventogenic cells, and spores, respectively, and μ_S, μ_D, μ_{lyses} the specific rate of solventogenic cell formation, spore formation and cell lyses, respectively.

The mass balance referred to each of the three classes of cells and extended to the reactor and the filtration unit reads:

Total cell $$W_X = W_X^A + W_X^S + W_X^D \quad (10)$$

Acidogenic cells $$W_X^A = \mu X_A - \mu_S X_A \quad (11)$$

Solventogenic cells $$W_X^S = \mu_S X_A - \mu_D X_S \quad (12)$$

where W_X is the total biomass production rate, W_X^A , W_X^S, and W_X^D the mass production rate of acidogenic cells, solventogenic cells and spores, respectively.

The combination of Eq. (2) referred to the acidogenic cells and Eq. (11) yields:

$$D_{OUT} = \mu - \mu_S \quad (13)$$

The main assumption of the proposed model is that under the operating conditions adopted, the specific rate of spore formation and cell lyses are smaller than the μ_S. In particular, the model was developed according to the following assumptions:
the specific rate of cell lyses negligible;
Flickinger and Drew (1999) reporte that the cell death under a wide interval of adverse environmental conditions was characterized by the specific death rate ranging between 0.0015 and 0.009 h^{-1}. As a first attempt, the model was developed setting $\mu_D = 0.0045 h^{-1}$ and a sensitivity analysis is reported at the end of the "Results and Discussion" section.
The specific growth rate may be estimated by means of the relationship propose by Napoli et al. (2011):

$$\mu = \mu_{max} \cdot \frac{C_L}{C_L + K_L} \cdot \left(1 - \frac{AA}{AA_{max}}\right)^{n_{AA}} \cdot \left(1 - \frac{BA}{BA_{max}}\right)^{n_{BA}} \cdot \left(1 - \frac{Ac}{Ac_{max}}\right)^{n_{Ac}} \cdot \left(1 - \frac{Et}{Et_{max}}\right)^{n_{Et}} \cdot \left(1 - \frac{B}{B_{max}}\right)^{n_B} \quad (14)$$

Metabolites

The fermentation process of *C. acetobutylicum* on lactose as regards acidogenic and solventogenic stages may be described by means of the reaction set reported in Table 1.

Reaction		**Ref.**	
L →8ATP+4AA+4CO2+8H2-4H2O	(1)	(a)	
L → 6ATP+2BA+4CO2+4H2	(2)	(a)	
L+2AA → 4ATP+2Ac+6CO2+4H2+2Et	(3)	(b)	
L+2AA → 4ATP+2Ac+6CO2+4H2+B+H2O	(4)	(b)	
L+2BA → 4ATP+2Ac+6CO2+4H2+B	(5)	(b)	
L → 4ATP+4CO2+B+2H2O	(6)	(b)	
L → 4ATP+4CO2+4Et	(7)	(b)	
L+3NH3+3(101/YATP)ATP → 3X	(8)	(a)	

Table 1. Lumped reactions of the Clostridium acetobutylicum fermentation on lactose (L, lactose; AA, acetic acid; BA, butyric acid; B, butanol; Et, ethanol; Ac, acetone)
(a) Napoli et al. (2012)
(b) Papoutsakis (1983)

The biomass in Eq. (8) of Table 1 was described as $C_4H_7O_2N$ according to the formula proposed by Zeng et al. (1996) for *C. butylicum*. Therefore, the biomass is characterized by: molecular weight MWX=101 $gmol^{-1}$; carbon fraction σ=0.475; reductance degree γ=4.
The molar balance referred to extracellular metabolites and extended to the reactor and the filtration unit reads:

Acetic ac. $$F_{AA} = f_1^{AA} - f_3^{AA} - f_4^{AA} \quad (15)$$

Butyric ac. $$F_{BA} = f_2^{BA} - f_5^{BA} \quad (16)$$

Butanol $$F_B = f_4^B + f_5^B + f_6^B \quad (17)$$

Ethanol $$F_{Et} = f_7^{Et} + f_3^{Et} \quad (18)$$

Acetone $$F_{Ac} = f_3^{Ac} + f_4^{Ac} + f_5^{Ac} \quad (19)$$

where F_i is the molar net rate of production/uptake of the i-species measured during the tests, and f in the molar rate of production/uptake of the i-species with respect to the reaction "n" of Table 1. In particular, "i" may be: Ac - acetone, AA – acetic acid, B

– butanol, BA – butyric acid, Et – ethanol. According to the metabolic reactions reported in Table 1, the following stoichiometric relationships were adopted:

$$f_4^{Ac} = f_4^{AA} \quad (20)$$

$$f_4^{Ac} = f_4^{AA} \quad (21)$$

$$f_4^{Ac} = f_4^{AA} \quad (22)$$

$$f_3^{Et} = f_3^{AA} \quad (23)$$

The model consists of 8 equations and 9 fluxes. The solution of the model requires a further relationship. A set of observation are hereinafter listed to support the assumptions adopted to assess the fluxes of the model:

a) the uptake of acids is active for all acids. Therefore, uptake reactions of all acids must be included in the final set of equations;

b) the acetic acid may produce both butanol and ethanol.

The adopted assumptions have been:

i) the reaction 3 may be neglected with respect to reaction 4 without any effect on the metabolite distribution. As a consequence, all acids are still re-assimilated. The possible alternative to neglect reaction 4 with respect to reaction 3 was also investigated. However, results did not change when reaction 4 is neglected and the reaction 3 is taken into account.;

ii) the molar ratio between butyric and acetic acids is constant and equals to 1.5 under acidogenic conditions (Napoli et al., 2009).

Taking into account the stochiometric relationships and the assumptions listed above, the flux model is simplified as follow:

Acetic ac. $$F_{AA} = f_1^{AA} - f_4^{AA} \quad (24)$$

Butyric ac. $$F_{BA} = f_2^{BA} - f_5^{BA} \quad (25)$$

Butanol $$F_B = \frac{f_4^{AA}}{2} + f_5^{B} + f_6^{B} \quad (26)$$

Ethanol $$F_{Et} = f_7^{Et} \quad (27)$$

Acetone $$F_{Ac} = f_4^{AA} + f_5^{BA} \quad (28)$$

Butyric ac./Acetic ac. ratio $$\frac{f_2^{BA}}{f_1^{AA}} = 1.5 \quad (29)$$

The conversion process is described by a set of six independent equations in the six variables $\left(f_1^{AA}, f_4^{AA}, f_2^{BA}, f_5^{BA}, f_6^{B}, f_7^{Et}\right)$. The production flux of acetic acid $\left(f_1^{AA}\right)$ and of butyric acid $\left(f_2^{BA}\right)$ are adopted to assess the acidogenic cell concentration according to the relationship proposed by Napoli et al. (2012):

$$X_A = \frac{Y_{ATP}\left(2 f_1^{AA} + 3 f_2^{BA}\right)}{\mu} \quad (30)$$

where Y_{ATP} is the ATP yield - expressed as dry mass of cells produce per mole of ATP generated- proposed by Bauchop and Elsden (1960).

The value of f_1^{AA}, f_4^{AA}, f_2^{BA}, f_5^{BA}, f_6^{B}, f_7^{Et}, X_A, are adopted to assess X_S, X_D and μs by resolving the set of Eq.s (15) trough (19).

The production rate of butanol referred to the mass unit of solventogenic cells (r_B) was assessed according to Eq. (31):

$$r_B = \frac{D\,B}{X_S} \tag{31}$$

Data of r_B were interpreted taking into account that lactose, acetic acid and butyric acid are substrate and butanol the inhibition product. In particular, the Monod-Boulton model proposed for process inhibited by product (Arellano-Plaza et al., 2007) was adopted.

$$r_B = r_{B,MAX}\left(\frac{C_L}{K_{L,B}+C_L}\right)\left(\frac{AA}{K_{AA}+AA}\right)\left(\frac{BA}{K_{BA}+BA}\right)\left(\frac{K_B}{K_B+B}\right) \tag{32}$$

where r_{BMAX} is the maximum specific production rate, K_L,B, K_{AA}, K_{BA} and K_B constants of the model.

Computation procedure

The value of r_{BMAX}, K_L, B, K_{AA}, K_{BA} and K_B were determined by a parametric inference procedure. Data of r_B measured during the continuous tests were processed by means of a regression tool of Sigma Plot®.

Results and discussion

Solvent production

Figure 3A-B and Table 2 report data measured during steady states of the fermentation carried out feeding the reactor with 50 g L^{-1} lactose bearing stream. The concentration of lactose (C_L), cells (X_{TOT}) and metabolites (acids and solvents) are reported in Fig. 3A-B as a function of the dilution rate at R set at 88%. The measured pH was 4.7 in all operating conditions adopted. As expected, the lactose conversion and the concentration of products (cells and metabolites) decrease with the dilution rate (time-space decreases). In particular, butanol concentration decreases remarkably with D. The butanol selectivity– reported in Table 2 - increased with D and it approached a constant value of about 0.90 g g^{-1}.

Figure 3C reports the specific productivity of butanol and solvents assessed for the tests reported in Fig. 3A-B. Both productivities increased with D. A double slope may be observed in the productivity vs. D data with a discontinuity at D≈0.1 h^{-1}: the slope at lower D is higher than that at higher D.

Table 3 reports the results of the two set of tests carried at a pre-set D (0.07 and 0.1 h^{-1}) and changing R. The pH was 4.7 in all operating conditions adopted. The butanol and the ABE concentration, the specific productivity of both butanol and solvents increase with R, for all values of D set. The increase of the specific productivities with D is confirmed in R adopted. The maximum specific productivity of both butanol and ABE was found at D = 0.1 h^{-1} and R = 95%: 0.50 and 0.56 g L^{-1} h^{-1}, respectively. Reported results are in good agreement with those reported by Napoli et al. (2009) operating a reactor-MF apparatus at D=0.04 1/h and R=100%. Indeed, Napoli et al. (2009) find butanol concentration of about 10 g L^{-1} when lactose conversion was over 90%. The comparison of results with recent investigations reported in the literature is made complex by the possible spectrum of conditions adopted by the research groups. Indeed, it is quite difficult to find tests carried out with the same strain, substrate, apparatus, and operating conditions. Results may be partially compared with those reported by Meyer and Papoutsakis (1989). The study focused on the substrate limitation. They operated an apparatus similar to that adopted in the present study but adopting glucose as carbon source (initial concentration ranging between 14 and 73 g L^{-1}). Reported tests may be grouped into series carried out at D

constant (0.2, 0.3 and 0.43) and R set at 0, 0.5, and 0.75. They found that both the concentration and the productivity of butanol increase with R.

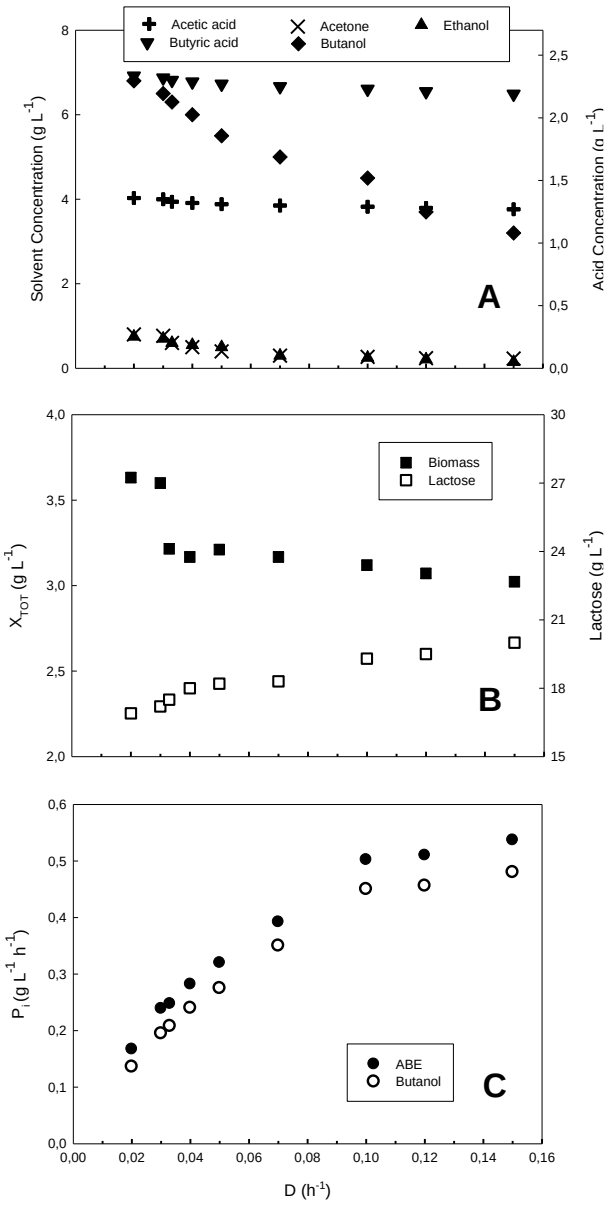

Figure 3. Data referred to steady states of continuous fermentation tests as a function of the dilution rate. R = 88%. A) Concentration of metabolites B) Concentration of lactose and biomass C) Solvent productivity.

The high performances reported by Meyer and Papoutsakis (1989) – butanol concentration 12.3 g L^{-1}, butanol productivity 2.83 g L^{-1} h^{-1} - than those reported in the present study may be interpreted taking into account the carbon source. Indeed, Yu et al. (2006) pointed out that “glucose is a preferred carbon source for this organism” with respect to lactose. It is interesting to compare present results with those reported by Zheng et al. (2013). They operate an apparatus similar to that adopted in the present study but using *C. saccharoperbutylacetonicum* N1-4 and xylose as strain and carbon source, respectively. Tests were carried out at fixed R=100% (the cell bearing stream out of the fermenter was absent) and at D increasing from 0.14 to 0.85. The data reported in the present investigation agree with those reported by

Zheng et al. (2013): the concentration of butanol decreases and the productivity of butanol increases with D.

D (h^{-1})	**ξ_L**	**Y_{B/C_L}**	**Selectivity ($Y_{B/C_L}/Y_{Sol/C_L}$)**
0.02	0.66	0.21	0.81
0.03	0.66	0.20	0.82
0.033	0.65	0.19	0.84
0.04	0.64	0.19	0.85
0.05	0.64	0.17	0.86
0.07	0.63	0.16	0.89
0.1	0.61	0.15	0.90
0.12	0.61	0.12	0.89
0.13	0.60	0.11	0.89

Table 2. Lactose conversion degree (ξL), butanol yield ($Y_{B/CL}$) and selectivity ($Y_{B/CL}/Y_{Sol/CL}$) of the continuous fermentation in the reactor-MF plant: effects of D at R 88%. Lactose concentration in the feeding 50 g L^{-1}.

Cell related kinetics

The concentration of acidogenic cells, solventogenic cells and spores were assessed according to the procedure described in previously section. Figure 4A reports the total cell concentration measured during the continuous fermentation tests as well as the calculated concentration of acidogenic cells, solventogenic cells and spores for continuous fermentation tests characterized by R=88% and D ranging between 0.02 and 0.15 h^{-1}.

The concentration of acidogenic cells increases linearly with D while the spore concentration decreases exponentially with D. The concentration of solventogenic cells is almost constant with D and increases of about 10% increase at low D. The analysis of results in Fig. 3A and of the metabolite concentration vs. D profiles (Fig. 3A-B) suggests that as D increases, the drift toward a less harsh conditions – low concentration of solvents and acids – promotes the presence of acidogenic cells at spore's expense. Figure 4B reports the specific growth rate μ and μ_S assessed according to the relationship proposed by Napoli et al. (2011) and by the model describe in the "Theoretical Framework" section, respectively. The plot of D vs. D and D_{OUT} [=D*(1-R)] vs. D are also reported to identify the operating window of the reactor. The μ was typically smaller than D and larger than D_{OUT}: the accumulation of acidogenic cells - $\mu > D_{OUT}$ - was prevented by the establishment of a cell population controlled by the equilibrium among acidogenic cells, solventogenic cells and spores. The μ_S vs. D was characterized by a maximum at D=0.05 h^{-1}. The analysis of μ_S and of concentration of acids and solvents (Fig. 3A) suggests that acids promote the solventogenic cell formation while solvents inhibit the formation. Cell concentration and specific growth rate assessed for tests carried out at constant D (0.074 and 1 h^{-1}) and at R ranging between 14% and 95% are reported in Table 3. The concentration of cells and of butanol increase with R for the adopted D. It is worth to note that the specific growth rate is larger than D at low R. However, under these conditions the high rate of the solventogenic cell formation – high value of both μ_S and X_A – prevents cell accumulation in the fermenter.

Operating conditions		Measured data				Calculated data						
D (h-1)	R %	L (gL^{-1})	B (gL^{-1})	B/ABE (-)	XTOT ($g_{DM}L^{-1}$)	PBut ($gL^{-1}\ h^{-1}$)	PABE ($gL^{-1}\ h^{-1}$)	X_A ($g_{DM}L^{-1}$)	X_S ($g_{DM}L^{-1}$)	X_D ($g_{DM}L^{-1}$)	μ (h^{-1})	μ_S (h^{-1})
0.1	14	41	2.8	0.94	1.29	0.28	0.29	0.43	0.82	0.04	0.269	0.180
0.1	54	35	3.4	0.95	1.82	0.34	0.35	0.64	1.08	0.09	0.149	0.097
0.1	88	19.3	4.5	0.90	3.12	0.45	0.50	1.04	1.56	0.52	0.040	0.027
0.1	95	15	5.0	0.89	4.55	0.50	0.56	1.10	2.17	1.28	0.031	0.023
0.07	14	34	3.8	0.95	1.44	0.26	0.27	0.38	0.97	0.084	0.198	0.146
0.07	54	30	4.0	0.95	2.06	0.28	0.29	0.51	1.33	0.21	0.113	0.085
0.07	88	18.3	5.0	0.89	3.16	0.35	0.39	0.74	1.60	0.82	0.037	0.028
0.07	95	12	5.6	0.87	4.70	0.39	0.45	0.81	1.91	1.98	0.025	0.020

Table 3: Main data of the continuous fermentation in the reactor-MF plant: effects of R at two values of D. Lactose concentration in the feeding 50 g L^{-1}.

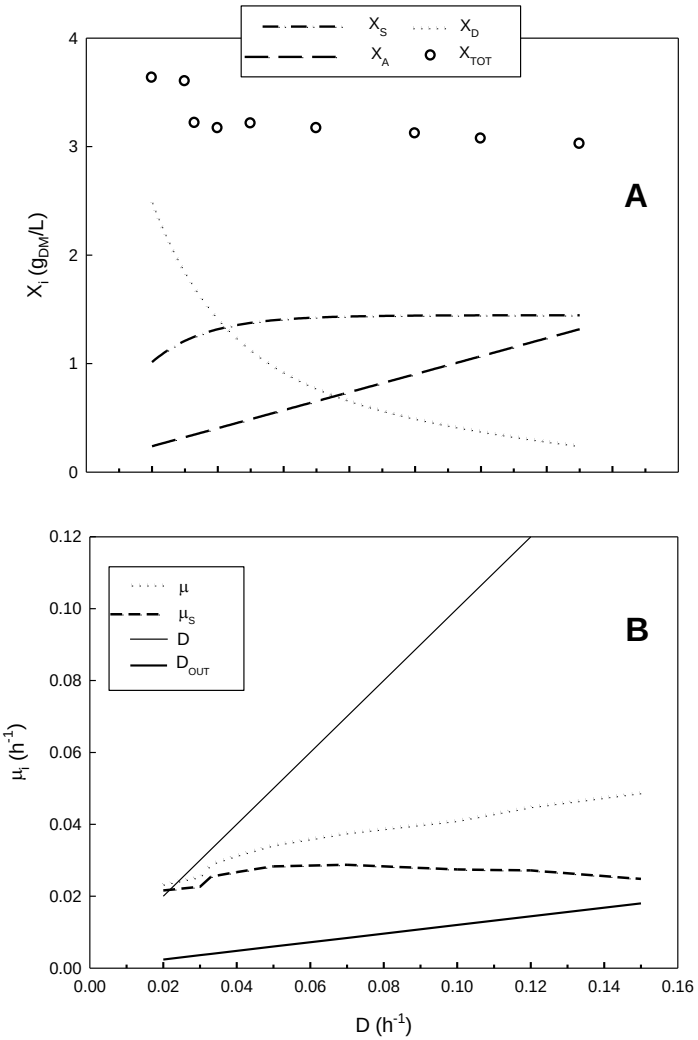

Figure 4. Data measured (points) and calculated (lines) for steady states of continuous fermentations as a function of the dilution rate. R = 88%. A) Concentration of acidogenic cells (X_A), solventogenic cells (X_S) and spores (X_D). B) Specific growth rate: μ_S is the plot of Eq.14 (Napoli et al., 2011).

Figure 5 reports D_{OUT} measured (Eq. 2) vs. D_{OUT} assessed (Eq. 13). Inspection of the parity plot indicates that the departure of model predictions from experimental data points is fairly small, typically within 10%.

Kinetics of butanol production

The production rate of butanol referred to the mass unit of solventogenic cells (r_B) – Eq. (31) - was calculated for all tests. Data regression according to Eq. (32) provided the assessment of the relationship constants. The results of the best-fit procedure are reported in table 4. $K_{L,B} \approx 0$ g L^{-1} suggested that the butanol production rate does not depend on the lactose. As a consequence, the conversion time of all lactose to butanol does not depend on the lactose concentration: reaction rate of zero order.

Figure 6 reports the parity plots of the measured r_B with the value assessed according to Eq. (32) and using model parameters reported above. The error between the model prediction and experimental data is typically within 10%. Therefore, the soundness of the proposed theoretical framework may be claimed.

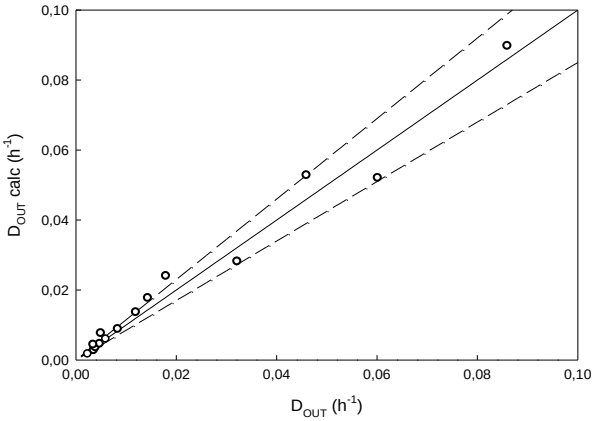

Figure 5. D_{OUT} calculated vs. D_{OUT} measured. Dashed lines : ±10% error.

Sensitivity analysis to the specific rate of spore formation

The results reported in the previous sections have been obtained setting the specific rate of spore formation (μ_D) at 0.0045h^{-1}. The sensitivity of the model parameters on the specific rate of spore formation is discussed hereinafter.

According to Flickinger and Drew (1999) μ_D ranges between 0.0015 and 0.009h^{-1}, in other terms the value of μ_D may range over an order of magnitude. Results of the model have been assessed setting μ_D at 0.0015 and at 0.009 h^{-1}. As regards the concentration of solventogenic cells and spores, the concentration typically changes less then 25% with respect to the value assessed at μ_D=0.0045 h^{-1}. Table 4 reports the results of the sensitivity analysis as regards the kinetic parameters of the specific butanol production rate. Except for $K_{L,B}$, the value of the kinetic model constants does not change remarkably with μ_D. $K_{L,B}$ departs from zero at low μ_D but it is still quite low to affect remarkably the butanol production rate for lactose conversion approaching unity.

	$r_{B,MAX}$ (g_B $g_{DM}^{-1}h^{-1}$)	K_L (g L^{-1})	K_{AA} (g L^{-1})	K_{BA} (g L^{-1})	K_B (g L^{-1})
μD = 0.0045 h^{-1}	4.7	≈ 0	0.8	0.25	0.48
Sensitivity with respect to μ_D					
μ_D = 0.0015 h^{-1}	2.6	5	0.1	0.05	0.48
μ_D = 0.0091 h^{-1}	5.9	≈ 0	0.8	0.25	0.48

Table 4. Kinetic parameters of the specific butanol production rate model (**Eq. 32)** and results of the sensitivity analysis with respect to μ_D.

Conclusions

The characterization of *C. acetobutylicum* DSM 792 on lactose during solventogenic phase was carried out in a CSTR equipped with a microfiltration unit. The role of the solventogenic cells in the butanol production rate was assessed. The butanol production rate was referred to the mass unit of solventogenic cells and not to all the biomass present in the broth (acidogenic/solventogenic cells and spores). The proposed model for the butanol production rate successfully took into account just the solventogenic cells. The model may be adopted for the design and the optimization of reactor systems characterized by a the heterogeneous cell population.

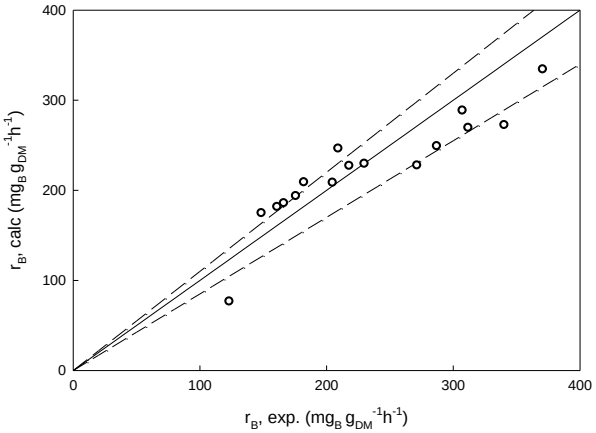

Figure 6. r_B calculated (Eq.32) vs. measured (Eq.31). Dashed lines : ±10% error.

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5.4 The support of the kinetics to the design of a continuous ABE fermenter

The design of a continuous reactor for ABE fermentation is based on the kinetics of the conversion process. The presence of a cell population (acidogenic cells, solventogenic cells, and spores) charcaterized by mutual interferences and the butanol production by means of one element of the cell population makes the design of a continuous reactor a complex task.

The overall kinetics identified in the present section of the PhD thesis and by Napoli et al. (2011, 2012) may be addressed to support the design and optimization of a fermenter. Indeed, the kinetic framework of the fermentation allows to assess/sumulate the conversion of the substrate (lactose/xylose), the production of acids and solvents. Just as memo, the specific growth rate and the butanol production rate for a generic substrate (concentration C_S) are reported in the following:

$$\mu = \mu_{\mathbf{max}} \cdot \frac{C_S}{C_S + K_S} \cdot \left(1 - \frac{AA}{AA_{\mathbf{max}}}\right)^{n_{AA}} \cdot \left(1 - \frac{BA}{BA_{\mathbf{max}}}\right)^{n_{BA}} \cdot \left(1 - \frac{Ac}{Ac_{\mathbf{max}}}\right)^{n_{Ac}} \cdot \left(1 - \frac{Et}{Et_{\mathbf{max}}}\right)^{n_{Et}} \cdot \left(1 - \frac{B}{B_{\mathbf{max}}}\right)^{n_B} \quad (1)$$

$$r_B = r_{B,MAX} \left(\frac{C_S}{K_{S,B} + C_S}\right)\left(\frac{AA}{K_{AA} + AA}\right)\left(\frac{BA}{K_{BA} + BA}\right)\left(\frac{K_B}{K_B + B}\right) \quad (2)$$

The development of a model to simulate a fermenter will ask for the selection of the flow configuration (CSTR, PFR, series of CSTR, etc.) and the choice between free cells and immobilized cells (biofilm). The selection of biofilm configurations will introduce extra phenomena into the conversion process such as mass transport to/from and intra the biofilm (Raganati et al., 2013).

The first step of the kinetic characterization has been provided. The next steps will ask further details.

CONCLUSIONS

The study carried out during the present Ph.D. program aimed at investigating the Acetone-Butanol-Ethanol (ABE) fermentation to produce butanol. The activities were articulated according to three paths:

i) feedstock market and techno-economic feasibility assessment of butanol production;
ii) biomass pretreatment;
iii) butanol production and characterization of the ABE fermentation process.

The activity was mainly carried out at Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale of the Università degli Studi di Napoli 'Federico II'. The stints at the "Enco" Engineering Consulting Company and at the "University of Western Ontario" Canada were functional to the progress of the activities and were successful to integrate the research activity carried out at the Dipartimento.

Feedstocks market and economic feasibility assessment

A survey of potential "waste biomass" for butanol production was proposed. Substrates investigated were: lignocellulosic biomass (dedicated coltures), agriculture residues, HSCB (Hight Sugars Content Beverages), cheese-whey (industrial waste water), waste food. The maximum butanol production rate from each biomass was estimated taking into account the feedstock availability rate, the average composition, and the butanol yield. The optimistic assessed butanol production was about 20 % of the European demand for fuel. The effective butanol production is lower of the optimistic assessment because the sugar conversion may be partial and a fraction of solvent may be lost during the recovery and concentration process.

The techno-economic feasibility of a butanol production process from lignocellulosic biomass was carried out. A potential flowsheet to produce butanol by conversion of a lignocellulosic biomass was simulated by means of the software Aspen Plus®. Particular attention was paid to the upstream process. **The comparison between the economical potential and the depreciation rate of the main fixed investments lefts room for the economic feasibility of the process**.

Biomass Pretreatment

A new class of solvents DES (Deep Eutectic Solvent) was investigated to obtain fermentable sugars from corncob. Tests were carried out adopting three different DES-mixtures: choline chloride and glycerol, choline chloride and imidazol, choline chloride and urea. 98% glucose and xylose enzymatic conversion in 10h after choline chloride and glycerol pretreatment was obtained.

Butanol production and characterization of the ABE fermentation process

The assessment of the kinetics of butanol production by *C. acetobutylicum* was carried out. Xylose and lactose were used as carbon sources. Acids production by acidogenic cells and butanol production by solventogenic cells were investigated using different reactor configurations: CSTR under controlled pH and CSTR with microfiltration unit respectively.

The cell population under solventogeneic conditions was also assessed under solventogenic conditions. In particular the fraction of acidogenic cells, solventogenic

cells, and endospores was assessed together the transition kinetics among the cell classes. The butanol production rate was assessed as a function of the concentration of the carbon source, acids and solvents. The rate depended on the xylose concentration and did not depend on the lactose concentration. The rate was referred to the solventogenic cells. The operating conditions for the optimal butanol production depended on the obiective: maximum butanol concentratiuon and maximum butanol production.
The potential application of the kinetics to the design of continuous fermenters was proposed. The complete development of the model applied to a specific reactor systems (e.g. biofilm packed bed) could be the aim a successive research program.

The results reported in the present PhD thesis are satisfactory and they are promising to develop the butanol production process by the fermentation route at industrial scale. The results also highlighted the success of the butanol production process asks for the optimization of the recovery and concentration of butanol from the fermentation broth (see also Liu and Fan, 2004; Napoli et al., 2012). In particular, the optimization of the fermentation coupled with a butanol recovery system could be the objective of a future research project.

Nomenclature

AA, Ac, B, BA, Et	Concentration of acetic acid, acetone, butanol, butyric acid, ethanol (g L^{-1})
AA_{max}, BA_{max}, Ac_{max}, B_{max} Et_{max},	Critical concentrations of acids and solvents (g L^{-1})
AA_{max}, BA_{max}, Ac_{max}, B_{max}, Et_{max}	Critical concentrations of acids and solvents (g L^{-1})
D_i	Dilution rate with respect to the stream "i" (h^{-1})
F^i	Molar net rate of production/uptake of the species " i " assessed experimentally
f^i_n	Molar rate of the production/uptake of the species " i " referred to the metabolic reaction n
K_{Xyl}, K_L, K_L,B	Constant (g L^{-1})
K_B	Inhibition constant (g L^{-1})
C_L	Lactose (g L^{-1})
C_{Xyl}	Xylose (g L^{-1})
μ	Specific growth rate (h^{-1})
n_{AA}, n_{BA}, n_{Ac}, n_{Et} and n_B	parameter of Eq. (14)
R	Recycle ratio
ξ_L	Lactose conversion degree
Y_{ATP}	ATP yield (g_{DM}/mol_{ATP})
Y_{ATP}^{MAX}	maximum ATP yield (g_{DM}/mol_{ATP})
$Y_{X/Xxyl}$	fractional yield of xylose in biomass (g_{DM}/g)
$Y_{BA/Xyl}$	fractional yield of xylose in butyric acid (g/g)
$Y_{AA/Xyl}$	fractional yield of xylose in acetic acid (g/g)
$Y_{X/A}$	mass ratio between biomass and total acids (g_{DM} g^{-1})
$Y_{A/ATP}$	ratio between total acids and ATP moles (g $molATP^{-1}$)
X	cell concentration (g_{DM} L^{-1})
X, X_A, X_S, X_D	Concentration of total cells, acidogenic cells, solventogenic cells and spores (g_{DM} L^{-1})
W_X, W^A_X, W^S_X, W^D_X	Mass flow rate of total cells, acidogenic cells, solventogenic cells and spores (g_{DM} $L^{-1}h^{-1}$)

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A. Procentese, F. Raganati, G. Olivieri, M.E Russo, M. De La Feld, A. Marzocchella. Renewable Feedstocks for Biobutanol Production by Fermentation. Submitted to Biomass & Bioenergy

Conference presentations

F. Raganati, **A. Procentese**, G. Olivieri, M.E. Russo, P. Salatino, A. Marzocchella. MFA Of *Clostridium acetobutylicum* Pathway: The Role Of Glucose And Xylose On The Acid Formation/Uptake. IBIC 2014.

F. Raganati, **A. Procentese**, G. Olivieri, P. Salatino, A. Marzocchella. Biobutanol Production from Hexose and Pentose Sugars. IBIC 2014.

A. Procentese, T. Guida, F. Raganati, G. Olivieri, P. Salatino, A. Marzocchella. Process Simulation of Biobutanol Production from Lignocellulosic Feedstocks. IBIC 2014.

A. Marzocchella, G. Olivieri, **A. Procentese**, F. Raganati, M.E. Russo, P. Salatino. Bioreactor development for process intensification: butanol production. IFIB 2013: Italian Forum on Industrial Biotechnology and Bioeconomy. 22-23 October, 2013, Napoli (IT).

A. Procentese, F. Raganati, G. Olivieri, A. Marzocchella. Butanol production from lignocellulosic biomass 16th Ontario-Québec Biotech Meeting – Toronto, May 15-16,2014.

A. Procentese, F. Raganati, G. Olivieri, M.E Russo, P. Salatino, A. Marzocchella. Continuous xylose fermentation by *Clostridium acetobutylicum* – kinetics and energetics issues under acidogenesis conditions 16th European Congress on Biotechnology- Edinburgh July 13-16, 2014.

Permanence at research structures in Europe

31 January –31 July 2014. Research group leaded by Lars Rehmann. Department of Chemical and Biochemical Engineering, “Western University” London-Ontario-Canada.

10 October – 17 October 2014 ESPCA 2014 - São Paulo Advanced School on the Present and Future of BIOENERGY. University of Campinas (Unicamp), Brazil.

Attended course/seminaries

1 **Viola Calabrò.** Functional genomic December 2012
2 **John Van Der Oost.** Prokaryotic innate immunity-discovery of a new player. April 13
3 **G. Marino, F. Pane, F. Pennacchio, R. Rao, G. Sannia: "**Advanced biotechnology". May-June 2013.
4 **Peter Götz:** Dynamic Processes in Microbial Metabolism. May 2013.
5 **Marina Mione** Zebrafish as model for cancer. May 2013
6 **L. Rehmann** "Biochemical Conversion of Biomass for the Production of Value Added Chemicals". June, 3 2013
7 **Dr Rajeev K Sukumaran and Dr Binod Parameswaran:** Training course: "Second Generation Bioethanol". October 2013.
8 **R. Lauri:** "Aspetti di sicurezza relative a processi industriali finalizzati alla produzione di biocombustibili e bioplastiche" January 2015.
9 **B. Pietrangeli:** "Biotecnologie per lo sviluppo sostenibile: applicazioni e sicurezza" January 2015.
10 **A. Fierro:** "Il granfalloon del bioetanolo di seconda generazione:il caso studio per la regione Campania". February 2015.
11 **A. Marzocchella, P. Salatino.** Corso di Formazione per PON-SOLTESS "Elementi di ottimizzazione economica di impianti di processo" (60 h).
12 **A. Marzocchella** Gestione e ottimizzazione delle linee di processo 6 CFU
13 **G. Toscano** Biotecnologie ambientali 6 CFU
14 **P. Netti** Ingegneria dei tessuti 4 CFU
15 **G. Olivieri** Processi biotecnologici 10 CFU
16 São Paulo Advanced School on the Present and Future of BIOENERGY (60h)