BUTANOL PRODUCTION BY FERMENTATION OF RENEWABLE FEEDSTOCKS

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INDEX

RIASSUNTO

Introduzione. L'attuale scenario energetico-ambientale ha determinato un rinnovato interesse nella produzione di Acetone-Butanolo-Etanolo (ABE) per via fermentativa. Interesse che è stato ulteriormente stimolato dalla considerazione che i recenti progressi registrati in vari settori scientifici rendono i processi fermentativi di produzione di ABE potenzialmente competitivi con i più consolidati processi dell'industria petrolchimica (Cascone, 2008). A tal fine, particolare rilevanza rivestono i progressi nel settore della biologia molecolare (con riferimento ai microrganismi in grado di produrre solventi), unitamente agli sviluppi dei sistemi reattoristici utilizzati per le bioconversioni e delletecnologie utilizzate per il recupero e concentrazione di solventi. Tra i vantaggi dei processi produttivi di ABE per via fermentativa va annoverata la possibilità di ottemperare alle direttive Europee che promuovono l'utilizzo di rifiuti industriali come materie prime. L'utilizzo dei rifiuti/reflui risulta fattibile accertato che alcune specie di batteri anaerobici del genere *clostridia* sono in grado di metabolizzare un'ampia gamma di carboidrati tipicamente presenti nei reflui dell'industria agro-alimentare, inclusi gli esosi, i pentosi e i polisaccaridi. Nonostante le acclarate potenzialità dei sistemi microbici nel produrre sostanze di interesse industriale, lo sviluppo di processi è limitato da due aspetti: i) carenza di informazioni riguardanti le equazioni costitutive del processo (crescita microbica, produzione di solventi, fattori di resa, selettività); ii) bassa velocità specifica di conversione che impone l'adozione di ingenti volumi di reazione per garantire elevate potenzialità produttive. Quest'ultima considerazione ha stimolato la ricerca di misure rivolte all'intensificazione di processo, tra le quali si annovera il confinamento dei batteri per la formazione di biofilm su supporti granulari e loro esercizio in reattori bi- e tri-fasici (Qureshi et al., 2000).

Scopo della tesi. Lo studio svolto durante il corso di DdR ha riguardato l processo di produzione ABE (Acetone-Butanolo-Etanolo) per via fermentativa. Il lavoro è stato svolto al Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale dell'Università degli Studi di Napoli Federico II.
Le attività sono state articolate in tre linee: i) caratterizzazione macroscopica del processo di fermentazione ABE in termini cinetici e di resa, utilizzando differenti risorse rinnovabili (e.g. biomasse lignocellulosiche, bevande ad alto contenuto zuccherino, siero del latte); ii) caratterizzazione del processo di fermentazione ABE in accordo all'analisi dei flussi metabolici (MFA) e a modelli cinetici dettagliati; iii) sviluppo di reattori a biofilm innovativi per la produzione d butanolo.

Processo di fermentazione ABE utilizzando diverse risorse rinnovabili. Caratterizzazione macroscopica in termini cinetici e di resa.
Lo studio ha riguardato la valutazione delle cinetiche e delle rese cellulari e dei metaboliti prodotti durante la fermentazione di:

a) singoli zuccheri (glucosio, mannosio, arabinosio e xilosio) rappresentativi dell'idrolisi di biomasse lignocellulosiche;
b) singoli zuccheri (glucosio, fruttosio e saccarosio) rappresentativi delle bevande ad alto contenuto zuccherino;
c) bevande ad alto contenuto zuccherino.

a) Sono state condotte prove di fermentazione batch di singoli zuccheri (glucosio, mannosio, arabinosio e xilosio), variando la concentrazione di ciascun nutriente utilizzato per la preparazione del terreno sintetico. I principali risultati sono:

- *C. acetobutylicum* è in grado di metabolizzare gli zuccheri tipicamente presenti nelle biomasse lignocellulosiche e convertirli in solvent;
- La concentrazione ottimale di coppia tampone K_2HPO_4/KH_2PO_4 è 0.25 g/L;
- L'aggiunta di $CaCO_3$ (concentrazione ottimale 5 g/L) al terreno di coltura aumenta sia il grado di conversione del substrato sia la concentrazione finale di solventi;
- La resa in butanolo e il rapporto molare butanolo/acetone aumentano in condizioni di limitazione di ferro per le fermentazioni di glucosio e mannosio;
- Tranne che per il glucosio, le prestazione della fermentazione – concentrazione massima di butanolo e massima velocità specifica di produzione di butanolo – migliorano all'aumentare della concentrazione di Mn^{2+}. Per il glucosio, le prestazioni della fermentazione presentano un massimo a concentrazione di $MnSO_4$ di 0.01 g/L;
- Mg^{2+} influisce positivamente sulla conversion di glucosio. Le prestazioni della fermentazione sono ottimizzate a concentrazione di $MgSO_4$ di 0.2 g/L.

L'analisi dei risultati delle prove di fermentazione batch condotte utilizzando i singoli zuccheri rappresentativi delle biomasse lignocellulosiche ha evidenziato che l'ottimizzazione del processo di produzione ABE richiede la selezione accurata del terreno di coltura, plausibilmene assortito con specie poco costose. La composizione del terreno di coltura tipicamente adottata per la fermentazione del glucosio non è risultata essere ottimale per tutti gli zuccheri investigati.

b-c) L'obiettivo di tale attività è stato duplice: i) proporre un possibile processo di smaltimento dei reflui prodotti dale industrie produttrici di bevande ad alto contenuto zuccherino; ii) produrre butanolo adottando materie prime che non sono in competizione con le risorse alimentari.

Sono state investigate quattro tipologie di bevande commerciali: due succhi di frutta (ananas e pera), due sciroppi (limone e mandorla), due soft drink e uno sport drink.

Tali bevande sono caratterizzate da elevate concentrazioni di zuccheri – tra 50 e 1000 g/L – e il loro smaltimento è un problema di primaria imortanza per le industrie del settore.

Il processo di conversione delle bevande ad alto contenuto zuccherino, in condizioni batch, è stato caratterizzato in termini di produzione cellulare e di metaboliti e in termini di conversione degli zuccheri. I principali risultati sono:

- *C. acetobutylicum* non è in grado di crescere adottando le bevande tal quali;
- *C. acetobutylicum* è in grado di crescere adottando sulle bevande addizionate (con yeast extract e sali). In condizioni ottimizzate la massima concentrazione di butanolo è di circa 10 g/L. Fruttosio e glucosio sono stati convertiti completamente alla fine del processo di fermentazione. Il saccorosio è solo parzialmente convertito.
- Le prove condotte con le bevande idrolizzate e addizionate sono caratterizzate da eccellenti risultati sia in termini di produzione di solventi (circa 14 g/L di butanolo) e sia di conversione degli zuccheri.

Tali risultati hanno dimostrato che le bevande ad alto contenuto zuccherino sono un substrato promettente per il processo di fermentazione ABE. Il processo investigato presenta un duplice vantaggio: ridurre i costi di smaltimento di tale tipologia di bevande e produrre butanolo da utilizzare come biocombustibile.

Caratterizzazione del processo di fermentazione ABE attraverso MFA e modelli cinetici.

L'analisi dei flussi metabolici (MFA) e un modello cinetico sono stati utilizzati per caratterizzare la fermentazione ABE. Sono state caratterizzate fermentazione batch in termini di serie temporale dettagliata di concentrazione dei metbaliti, celle e substrato. La concentrazione iniziale degli zuccheri investigati è stata fissata a 60 g/L. I dati di concentrazione sono stati elaborati per valutare I flussi metabolici e per implementare il modello cinetico. In particulare:

- La metodoligia MFA è stata implementata per investigare il ruolo dei singoli passi di reazione del pathway metabolico di *Clostridium acetobutylicum* per convertire in butanolo gli zuccheri di riferimento (glucosio e xilosio) degli idrolizzati di biomasse lignocellulosiche. I risultati delle prove di fermentazione batch di glucosio e xilosio sono stati utlizzati per valutare i flussi metabolici. La matrice stechiometrica del modello è caratterizzata da una singolarità che impedisce la determinazione unica del set di flussi metabolici. La singolarità è stata superata ricorrendo al vincolo proposto da Desai et al. (1999) di fissare il rapporto dei flussi di consumo di acido acetico e di acido butirrico. L'analisi dei flussi metabolici ha evidenziato il ruolo degli enzimi coinvolti nella formazione degli acidi. In particulare, sono stati evidenziati gli effetti della fonte di carbonio sulla rilevanza di ciascun step reattivo nel pathway di formazione/conversione degli acidi. Si è rilevato che il pathway di formazione del butirrato gioca un ruolo fondamentale sia nella produzione e sia nella conversione del butirrato senza formazione di acetone.
- Un modello cinetico della fermentazione ABE mediante *Clostridium acetobutylicum* DSM 792 è stato svilupato utilizzando il simulatore di network biochimici COPASI. L'effetto del substarto è stato studiato implementando il modello con divesi zuccheri: glucosio, mannosio, fruttosio, saccarosio, lattosio, xilosio e arabinosio. Il pathway metabolico è stato adeguato in relazione ai diversi zuccheri investigati. In particulare, le reazioni del pathway di Embden-Meyerhof-Parnas (EMP) sono state adottate per gli esosi e per i disaccaridi, le reazioni del pathway dei pentoso fosfati sono state adottate per i pentosi. Il modello è stato prima implementato per il glucosio, quindi si sono valutati i parametric cinetici degli altri zuccheri. Il modello proposto ha dato risultati soddisfacenti per ognuno degli zuccheri investigati: il coefficiente di correlazione (r^2) tra le serie temporali delle concentrazioni misurate e calcolati è contenuto tra 0.87 e 0.925.

Sviluppo di reattori a biofilm innovativi per il processo di produzione ABE

Lo studio è stato finalizzato alla valutazione del processo di produzione di butanolo da biofilm di *C. acetobutylicum.* L'attività ha riguardato la progettazione, la messa a punto e l'esercizio di un reattore a letto fisso con biomassa immobilizzata. Il reattore è stato alimentato con siero del latte. Le condizioni operative sono state selezionate al fine di massimizzare la produttività di butanolo. In particolare, l'attività è stata articolata in accordo alle seguenti fasi:

- selezione di una procedura di pre-trattamento del siero del latte e realizzazione di prove di fermentazione batch volte a valutare la migliore strategia di sterilizzazione;
- progettazione e messa a punto di un reattore a letto fisso con biomassa immobilizzata;
- esercizio del reattore in condizioni continue.

La conversione del siero del latte è stata caratterizzata in termini di produzione dei metabolite, conversione del lattosio e massa di biofilm. Le prove sono state condotte a velocità di diluzione (D) variabile tra 0.54 h^{-1} e 0.94 h^{-1}. I risultati migliori si sono ottenuti a D=0.54 h^{-1}: produttività di butanolo 2.7 g/Lh, concentrazione di butanolo 4.93 g/L, resa in butanolo rispetto al carboidrato convertito 0.26 g/g, selettività dei solventi (intesa come rapporto tra concentrazione di butanolo e concentrazione totale di solventi) 82%$_w$.

I buoni risultati ottenuti dall'esercizio del reattore a letto fisso con biomassa immobilizzata hanno sostenuto lo sviluppo, progettazione e messa a punto di una configurazione reattoristica innovativa: "carosello" di reattori a biofilm a letto fisso. I reattori sono disposti in serie con periodica rotazione dell'alimentazione ad uno dei reattori. È stato sviluppato un modello matematico di supporto alla configuarzione reattoristica adottata.

SUMMARY

The study carried out during the present Ph.D. program aimed at investigating the Acetone-Butanol-Ethanol (ABE) production process by fermentation. The work was carried out at the Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale of the University of Naples 'Federico II'. The activities were articulated according to three paths: i) the characterization of the ABE fermentation process as regards kinetics and yields using different renewable resources (lignocellulosic biomass, high sugar content beverges and cheese whey); ii) the characterization of the ABE fermentation process according to the metabolic flux analaysis (MFA) and to the dynamic kinetic models; iii) the development of innovative continuous biofilm reactor for the ABE production.
A commercial clostridia strain was investigated. *Clostridium acetobutylicum* DSM 792 was selected for its ability to produce ABE with satisfactory selectivity towards the butanol.

The ABE fermentation process by adopting renewable resources. Characterization in terms of kinetics and yields.

The study was aimed at the assessment of both the kinetics and the yields of cell growth and metabolites produced during the fermentation of:

- sugars representative of hydrolized lignocellulosic biomass fermentation (glucose, mannose, arabinose and xylose);
- sugars representative of high sugar content beverages (glucose, fructose and sucrose);
- High sugar content beverages.

Batch cultures of free *C.acetobutyicum* cells were inveastigated. Tests were focused on the preliminary characterization of the fermentation with the aim of highlighting the relevant features of the process. The fermentation was characterized in terms of kinetics and yields under a wide interval of operating conditions (substrate concentration, nutrient concentrations, …).

Characterization of the ABE fermentation process. MFA and dynamic kinetic models.

The MFA and the kinetic dynamic methodology were adopted to characterize the ABE fermentation. Selected batch fermentation tests were carried out with the aim to charcaterize the time-evolution of the concentration of substrate, cells and metabolities. Tests were carried out at initial sugar concentration 60 g/L. The time-series of concentration were processed according the MFA and the kinetic model methodology. In particular:

- the MFA was adopted to investigate the role of the main reaction steps of the *Clostridium acetobutylicum* metabolic pathway to convert reference sugars (glucose and xylose) of hydrolyzed lignocellulosic biomass into butanol. Results of batch fermentation tests carried out using glucose and xylose as carbon source were adopted for the flux assessment. The stoichiometric matrix of the model was characterized by a singularity that prevented the assessment of a unique set of fluxes of the primary metabolic activity. The non linear constrain proposed by Desai et al. (1999) relating the acetate and butyrate uptake fluxes was adopted to solve the model equation set. The MFA was proposed with reference to glucose and xylose as carbon source. The comparison of the assessed fluxes suggested the role of each reaction step as a function of the carbon source investigated.

- A kinetic dynamic model of acetone–butanol–ethanol (ABE) production by *Clostridium acetobutylicum* DSM 792 was proposed using the biochemical networks simulator COPASI. Effects of substrate were studied implementing the model with different sugars: glucose, mannose, fructose, sucrose, lactose, xylose and arabinose. If necessary, the metabolic pathway was modified according to the specific sugar. In particular, the Embden-Meyerhof-Parnas (EMP) pathway equations were used for hexose and disaccharide sugars while the pentose phosphate (PP) pathway equations were used for pentose sugars.

Development of innovative continuous biofilm reactor for the ABE production.

The study was aimed at the assessment of the butanol production in a *C. acetobutylicum* biofilm reactor. The activity aimed at the butanol production regarded the design, set-up and operation of a biofilm fixed bed reactor. Unsupplemented cheese whey was adopted as renewable feedstock. Operating conditions of the continuous tests were selected to maximize the butanol production and butanol selectivity. In particular, main activities were:

- The selection of a pre-treatment process of the cheese-whey coupled with fermentation tests carried out under batch conditions to assess effects of the pre-treatment on fermentation performances;
- Tests with the pre-treated cheese whey to characterize the fermentation process in terms of butanol production and butanol selectivity;
- Design, set-up, and optimization of a fixed bed biofilm reactor for cheese whey conversion;
- Tests with the biofilm reactor under continuous conditions.

The success of operation of the Packed Bed Reactor (PBR) has fuelled the development of an innovative continuous biofilm reactor configuration: “carosello” of packed bed biofilm reactors connected in series. The reactor system has been equipped with a device to switch the feeding at the reactor according to a pre-set sequence. A mathematical model to support the bioreactor system design was developed.

1 SCIENTIFIC BACKGROUND AND BIOTECHNOLOGICAL CONSEQUENCES

1.1 Introduction

The economic scenario characteristic of the beginning of the third millennium revives the interest in strategy for bioconversion of industrial wastewaters in biofuels and bulk chemicals. The objective of these strategies is threefold: i) to reduce the impact of anthropic processes on the environment; ii) to produce bulk-chemicals by eco-sustainable processes; iii) to diversify the fuel sources. The development of processes using biocatalyst systems – microorganisms or enzymes – is based on their ability to convert a wide spectrum of organic substrates - naturals and synthetics - even recalcitrant compounds. Even though the literature reports several witnessing of the potential of the cited biocatalyst systems, limited information is available to support the design and the operation of industrial plants, economically competitive. It should be pointed out that bio-based processes are characterized by low specific conversion rates. As a consequence, huge reactors are required for processes characterized by high throughput. Therefore, the development of industrial scale bio-based processes asks for innovative technologies aimed at intensified operations.

1.2 Background on Biobutanol

Butanol is a four carbon alcohol (C_4H_9OH). There are four structural isomers of which 1- butanol (n-butanol, Fig.1) is the most important commercial isomer. This isomer occurs in nature and it is primarily used industrially as a solvent or component in surface coatings. Butanol can also be used as fuel in internal combustion engines. The features of butanol are more attractive than those of the ethanol because the longer hydrocarbon chain causes it to be fairly non-polar. Butanol can be produced from both biomass ("biobutanol") and fossil fuels ("petrobutanol") without any difference of the chemical properties.

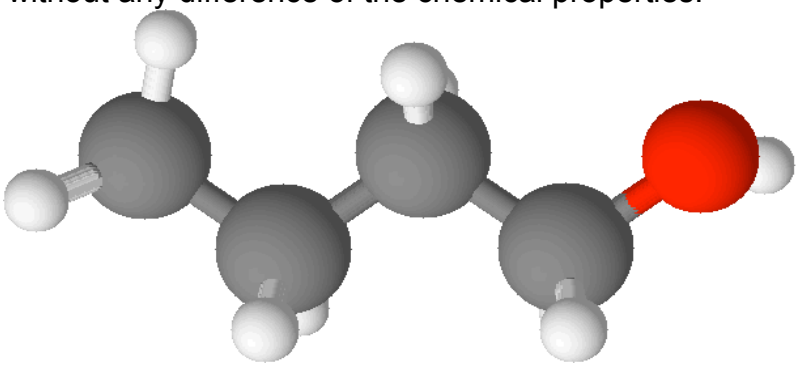

Fig.1: space filling model of 1-butanol.

1.2.1 Production History

Acetone was produced from wood up to World War I. The supply of wood became insufficient at the start of the war because acetone demand increased in line with the manufacture of cordite, a cartridge and shell propellant in which acetone was an essential ingredient. The Russian chemist C. Weizmann developed the ABE (acetone, butanol, and ethanol) fermentation process at Manchester University. In

1912 he isolated a strain which was later known as Clostridium acetobutylicum, and ran the first production plant for acetone production from starch (Dürre, 1998; Antoni et al., 2007). The fermentation process was about to be abandoned after the armistice in 1918, seeing that there was no further demand for acetone. There was, however, an increasing demand for butanol after the war. The rapidly expanding automobile industry required quick-drying lacquer which would give a good finish to car bodies (Jones and Woods, 1986). This resulted in a demand for some suitable solvent and it was found that butanol and its ester, butyl acetate, were ideal solvents for these lacquers. Between 1924 and 1927 new butanol production plants were built, and the isolation of molasses-fermenting strains increased plant capacity by 60% (Dürre, 1998). 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 to ethanol fermentation (Dürre, 1998). As the petrochemical industry evolved during the 1960s, the production of acetone and butanol by fermentation had virtually ceased. Cost issues, the relatively low-yield and sluggish fermentations, as well as problems caused by end product inhibition and bacteriophage infections, meant that biobutanol could not compete on a commercial scale with butanol produced synthetically (Brekke, 2007). Moreover, the molasses quality was decreasing due to improved sugar processing technology, and the price of molasses also increased seeing that it was used as a additive animal feeds (Zverlov et al., 2006). It was only in the USSR, China and South Africa that production continued. The plant in South Africa was closed in 1982 (Jones and Woods, 1986), and as the USSR disintegrated during the 1990s, their biobutanol production stopped (Antoni et al, 2007). In China, solvent fermentation was stepped down to complete closure only in 2004 (Chiao and Sun, 2007). Today most n-butanol is produced chemically from petroleum sources by either the oxo process starting from propylene (with H2 and CO over a rhodium catalyst), or the adol process starting from acetaldehyde (Brekke, 2007).

1.2.2 Research and Developments

There are a number of factors which stimulate the interest and funding for the research and development of biobutanol production. These include the current instability of oil supplies from the Middle East, a readily available supply of renewable agriculturally based biomass, and the call for reduction of greenhouse gas emissions.

Ultimately, a revival of the ABE fermentation process is dependent on favorable economic conditions relative to petrochemical-based processes (Ezeji et al., 2004a). In the early 1970s, the rising cost of petrochemicals combined with the energy crisis resulted in renewed interest in ABE fermentation. During the 1980s and 1990s there were tremendous progress in the development of genetic systems for the solventogenic Clostridia, which would allow for the development of strains with improved fermentation characteristics (Ezeji et al., 2004a).

Despite these developments there were still three major drawbacks to overcome before an economically competitive biological process could be reintroduced (Dürre, 1998):

- The high cost of the substrate.
- The low product concentration and productivity in fermentation due to end-product inhibition (16-18 g/L due to solvent toxicity).
- The high product recovery cost (product is very dilute and distillation has been used in the past).

During the past decade a hyper-butanol-producing strain has been developed as a result of the application of modern molecular techniques and genetic manipulation to the solventogenic Clostridia (Ezeji et al., 2007a). Experimental and computational engineering efforts have also led to improved fermentation techniques, downstream processing, and process integration. All these developments resulted in a significant increase in biobutanol concentration, yield and recovery.

A continuous fermentation pilot plant operating in Austria in the 1990s introduced new technologies and proved economic feasibility with agricultural waste potatoes (Nimcevic and Gapes, 2000). The Austrian plant helped bridge the skill gap between the termination of the US, USSR and South-African production and the recent renewal of production (Antoni et al., 2007).

In 2005, David Ramey drove a 13-year-old Buick across the United States, fuelled by pure butanol. Compared to gasoline, the consumption increased by 9%, but emissions of CO, hydrocarbons and NOx were reduced substantially. His company, Environmental Energy, Inc. (EEI), is planning to produce Butyl Fuel™ via a newly developed fermentation process involving two Clostridia species (Ramey and Yang, 2004). While this is a fairly small enterprise, there is a great market opportunity and larger companies, as well as oil companies, have started developing biobutanol.

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, June 20). They claim that their technology will be competitive as long as the crude oil price remains above 80 $ per barrel (Scott and Bryner, 2006).

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, June 20).

The owner of Virgin Atlantic, Richard Branson, is currently in the process of funding his own biomass to butanol fuel production plants (Oceanethanol, 2007, February 21). 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).

Biobutanol fermentation technology has been rapidly changing. It is suggested that future research might focus on the development of second-generation cultures which produce total ABE in the order of 25-33 g/L. Another approach where industrial progress could be made involves the recovery of fermentation by-products (large waste water streams, cell mass, CO_2 and H_2) for more profits, i.e. development of a biorefinery concept. These advances will help a fermentation-based biobutanol industry to compete effectively with petrochemical derived butanol (Ezeji et al., 2007a).

1.2.3 Industrial Importance

Butanol is a key bulk chemical with a wide range of industrial applications. Most of the worldwide production is converted into methacrylate esters and acrylate. Other main derivatives include glycol ethers and butyl acetate, while derivatives with minor uses are amino resins and n-butylamines. Applications, chemicals and products that use butanol include solvents (paints, varnishes, resins, gums, dyes, camphor, vegetable oils, fats, waxes, shellac, rubbers and alkaloids), plasticizers (to improve plastic material processes), coatings (as a solvent for a variety of applications, such as curable lacquers and cross-linked baking finishes), chemical intermediate or raw

material (for producing many chemicals and plastics, including safety glass, hydraulic fluids and detergent formulations), textiles (as a swelling agent and manufacturing garments from coated fabric), flotation agents, cleaners, floor polishes, cosmetics (including eye makeup, foundations, lipsticks, nail care products, personal hygiene products and shaving products), drugs and antibiotics, hormones, and vitamins (Dow, 2006).

1.2.4 Butanol as a fuel

A relatively new, but very important application is butanol as a biofuel. Butanol has several advantages over ethanol as a fuel component. It is less hygroscopic. In blends with diesel or petrol, butanol is less likely to separate from this fuel than ethanol if the fuel is contaminated with water. It is also less corrosive and more suitable for distribution by existing gasoline structures. The Reid vapour pressure of butanol is 7.5 times lower than that of ethanol, making it less evaporative/explosive (Bohlmann, 2007). Table 1 reports properties of common (bio)fuels and butanol.

Tab. 1. Liquid fuel features.

Characteristic	Gasoline	Butanol	Ethanol	Methanol
Formula	C_4-C_{12}	C_4H_9OH	CH_3CH_2OH	CH_3OH
Boiling Point (°C)	32-210	118	78	65
Energy Density (MJ/kg)	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/kg)	0.36	0.43	0.92	1.20

Processing the difference in energy densities reported in Table 1, a gasoline engine will theoretically have about 10% higher fuel consumption when run with biobutanol. However, tests with other alcohol fuels have demonstrated that the effect on fuel economy is not proportional to the change in energy density, and the effect of butanol on fuel consumption is to be assessed yet.

Compared to ethanol, butanol can be mixed at higher ratios with gasoline for use in running cars without any retrofit because the air-fuel ratio and the energy content are close to those of gasoline. Alcohol fuels, including butanol and ethanol, are partially oxidized and therefore need to run at richer air mixtures than gasoline.

Standard gasoline engines in cars can adjust the air-fuel ratio to accommodate variations in the fuel, but only within certain limits depending on model of the car. If the limit is exceeded by running the engine on both pure butanol and gasoline blend with a high percentage of butanol, the engine will run lean, a condition which can critically damage components (Smith and Workman, 2007). Butanol is considered substantially similar to gasoline for blending purposes and is certified by the U.S. Environmental Protection Agency as a blending agent up to 11 percent. Environmental Energy Inc. is a U.S. company with a patent for biobutanol production. It maintains that butanol can be used as a total replacement for gasoline without any modifications to car engines (Brekke, 2007).

In general, it is considered that the combustion process of biofuels have zero net carbon emissions due to its production from renewable agricultural feedstocks. Disadvantages of butanol with resepect to ethanol are higher viscosity and lower octane rating. A fuel with lower octane rating is more prone to knocking (extremely rapid and spontaneous combustion by compression) and reduces the engine

efficiency. Knocking can also cause engine damage. Butanol is also more toxic than ethanol.

1.2.5 The interest of the scientific community and of the industries

Fig.2 and Fig.3 report a world map with the density of the research groups and industrial enterprises interested in butanol production. It may be noted that the spread of industrial enterprises is still low. It should be pointed out that two large companies - BP and DuPont - have founded a joint venture to produce butanol. Some large plants are scheduled to be built up in Brazil.

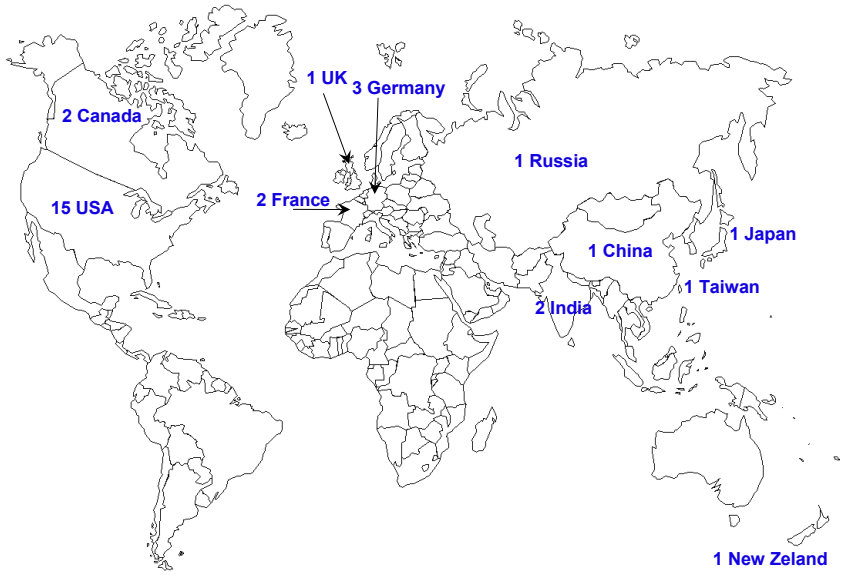

Fig. 2: The density map of research groups in the world interested to produce butanol or to investigate *Clostridia.*

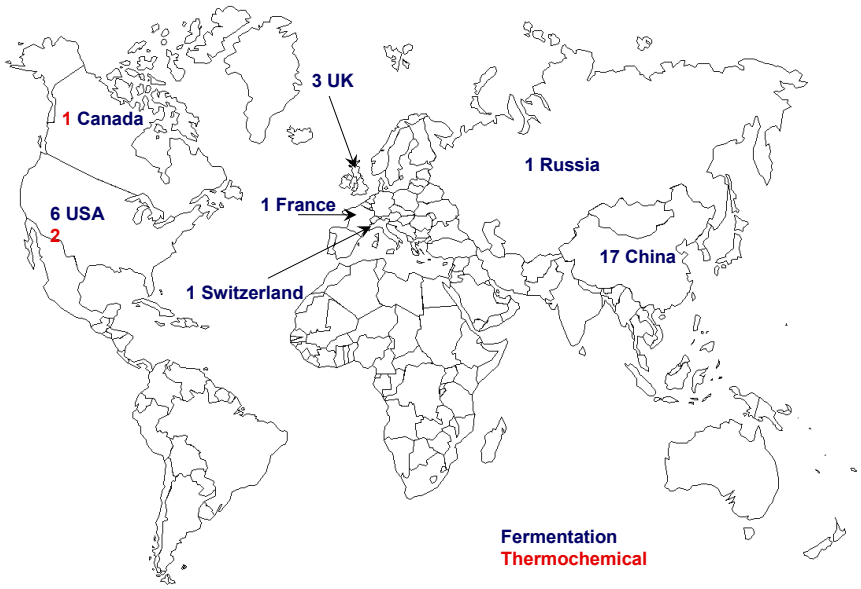

Fig. 3: The density map of enterprises in the world involved to produce butanol. Red numbers indicate the enterprises producing the butanol via the thermochemical route.

1.3 Literature Study On Fermentative Butanol Production

1.3.1 Substrates and Pre-treatment

The high cost of conventional substrates and 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). On one hand, the high carbohydrates content of some wastewater streams, e.g. the agro-industrial effluents, asks for expensive wastewater treatments. On the other hand, the use of these wastewaters as substrate can be a valid alternative to conventional-expensive feedstocks.

Apple pomace is a solid agricultural waste containing approximately 10%w carbohydrates (fructose, 67%, glucose, 23%, and sucrose, 10%). Voget et al. (1985) adopted apple pomace as feedstock in a fermentation process to produce butanol. They reported a yield of butanol between 1.9 and 2.2% of fresh apple pomace and a conversion of about 80-84% of the sugars. The residue recovered after butanol separation could be an excellent animal feed.

Marine algal biomass is considered to be an excellent fermentation substrate. Moreover, it is a potentially large renewable resource (Nakas et al., 1983). Results available in literature regarded mainly the conversion of a halophilic microalgae Dunaliella by *C. pasteurianum*. Nakas et al. (1983) pointed out that the microalgae were successfully converted and the final solvent concentration was about 16 g/L, provided a 4% glycerol medium integration. Particularly relevant was the absence of acetone among the solvents, typically produced during the *C. acetobutylicum* fermentations of molasses or starch. The solvent mixture was rich in butanol and 1,3-propanediol. Nakas et al. (1983) also pointed out that the solvent production was not inhibited by the salt present in algal concentrates. However, they suggested that the performance of industrial processes may improve if the salt concentration is decreased. As an alternative, the isolation of solvent-producing clostridia salt-tolerant would be wished.

Lignocellulosic material is the most abundant renewable resource (Lopez-Contreras, 2003). A large fraction (20-40%) of lignocellulosic biomass is hemicellulose (Flickinger and Drew, 1999; Fig.4), with D-xylose being the major component. *C. acetobutylicum* is capable of utilizing all of the prevalent sugars present in wood hemicellulose and cellulose hydrolysates (Ezeji et al., 2007a-b).

Cellulose exists in different forms characterized by a spectrum of degrees of polymerisation and molecular weight (Jacques et al., 2003). Different from cellulose, hemicelluloses are made up of shorter heteropoly saccharide chains that consist of mixed pentosans and hexosans. These carbohydrates are soluble, therefore susceptible to enzymatic breakdown. The main components of the arabinoxyl - a backbone of the hemicelluloses - are D-xylose and L-arabinose while the side chains are primarily composed of D-glucose, D-glucuronic, D-mannose and D-galactose. Glucuroxylan is the major constituent of hardwood hemicellulose, and glucomannan of softwoods (Jacques et al., 2003).

The methods utilized in lignocellulosic biomass pretreatment processes strongly depend on the structure of the raw material utilized.

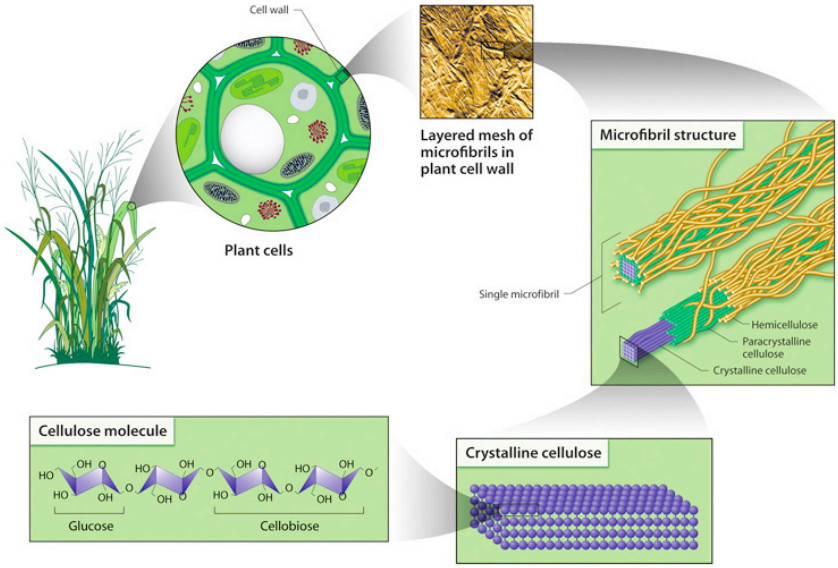

Fig.4: cellulose and hemicellulose in the microfibrils of a plant cell wall; [Genomics; 2007].

Pretreatment of lignocellulosic materials prior to utilization is a mandatory step in the biomass-to-butanol conversion processes. The objective of the pretreatment is to render biomass components more accessible to both chemical and enzymatic hydrolysis for efficient bioconversions. Main goals of the pretreatment are: i) to break and to remove the lignin sheath; ii) to remove and to separate the hemicellulose from cellulose; iii) to decrease the crystalline feature of the cellulose; iv) to increase the accessible surface area; v) to increase the pore size to facilitate the penetration of hydrolysis agents. Several processes have been developed for the treatment of various typologies of biomass. In general, the agricultural residues are easier to be treated than hardwood materials. Typically, softwood biomasses are recalcitrant because of the relatively high lignin content.

The spectrum of technology adopted for the pretreatment of biomasses is quite wide. The susceptibility of lignocellulosic components to successive enzymatic hydrolysis and microbial conversions depends on the technology adopted. The pretreatment processes must be efficient, cost effective, and environmental friendly. Main features of lignocellulosic pretreatments are reported in the following. The large part of the pretreatment technologies economically feasibile include a combination of mechanical size reduction, alkali swelling, acid hydrolysis, steam and other explosion techniques to disrupt the lignocellulosic fibers, e.g. exposure to supercritical fluids. The processes based on irradiation, strong mineral acids, cellulose solvents, and concentrated alkali chemicals are not suitable methods for the pretreatment of lignocellulosic materials because they are very expensive. Biological methods, e.g. processes adopting “white rot” fungi to disaggregate the lignin structure and to release the cellulose-hemicellulose components, are promising but the low conversion velocity requires more studies to increase the process throughput.

The ideal technology for the lignocellulosic-biomass pretreatment includes the dissolution of the solid materials into aqueous stream (Mussatto and Roberto, 2004). Many cellulose solvents at high concentrations can penetrate into the cellulose crystalline structure to dissolute the solid phase. The cellulose-derived matter can be readily hydrolyzed to glucose by cellulase either in situ or during upstream processes (Cao et al., 1994). Although cellulose solvents are powerful-solubilization agents, they are toxic, corrosive, and hazardous; moreover these treatments can result in the

formation of microbial inhibitors that are harmful to the ABE fermentation. Inhibitory compounds include furfural, hydroxymethyl furfural (HMF), and acetic, ferulic, glucuronic, ρ-coumaric acids (Ezeji et al., 2007b).

Economic considerations limit the development of processes adopting the described ideal technology to the industrial scale. Recently, particular attention has been paid to the direct utilization of lignocellulosic biomass. The individual components are separated and the cellulose is saccharified by enzymatic hydrolysis (Marchal et al., 1984). The sugars derived from both the cellulose and the hemicellulose may be properly fermented for the production of acetone and butanol.

In a study by Ezeji et al. (2007b), representative sugars present in lignocellulosic biomass were tested to determine their fermentability with *Clostridium beijerinckii* BA101. The sugars that were tested are glucose, xylose, cellobiose, mannose, arabinose, and galactose. Glucose served as the control for the experiment and produced an ABE concentration of 17.8 g/L with a productivity of 0.30 g/Lh. Rapid fermentations were observed with the other sugars as well, with productivities ranging from 0.23-0.32 g/Lh.

The ability of *Clostridium beijerinckii* BA101 to utilize mixed sugars (hexoses and pentoses) for ABE production was also tested, and it was found that mixed sugars can be metabolized simultaneously, although the rate of sugar utilization is sugar specific. The order of preference for utilization is glucose > xylose > arabinose > mannose. Fermentation time is longer when sugar mixtures are adopted as substrate than when pure glucose is adopted (productivity decreased to 0.21 g/Lh).

Corn fiber is a renewable resource available at significant rates from the corn dry and wet-milling industries. Approximately $4.7 \cdot 10^6$ dry tons of corn fiber is produced annually in the United States (Ebener et al., 2003; Ezeji and Blaschek, 2008). Typically, corn fiber is about the 10%w of corn and may be converted for more than the 66%w (about 5%w of the corn) in fermentable sugars. Although researches on genetics, fermentation, upstream processing, and downstream processing have progressed significantly, the clostridia are not able to efficiently hydrolyze fiber-rich agricultural residues. Therefore, agricultural biomass must be hydrolyzed in advance in simple sugars, using economic technologies. Dilute sulfuric-acid pretreatment can be applied to agricultural residues to perform hydrolysis. Unfortunately, a complex mixture of microbial inhibitors is generated during acid hydrolysis (e.g. hydroxymethyl furfural, and acetic, ferulic, glucuronic, r-coumaric acids, etc.) (Varga et al., 2004).

Sulfite waste liquors from the pulp and paper industries contain glucose, xylose, and arabinose and have been investigated as alternative substrates for ABE fermentation. Wiley et al. (1941) showed that satisfactory yields of solvents were obtained when the sulfur dioxide, lignin, and excess calcium were removed from the sulfite liquor.

Cheese whey has attracted interest as an alternative substrate for ABE fermentation because of its disposal issues (high BOD and COD), lactose content (about 5%w), and availability in many countries. About $13.6 \cdot 10^6$ ton/year of cheese whey is currently available in the U.S.. In Italy the long tradition in cheese production is translated in a cheese whey production of about 10^7 ton/year, and over 30% of dairy industries are located in Campania region. The typical composition of the cheese whey is: lactose 5%w; protein 0.67%w; ash 0.50%w; other solids 0.33%w; water 93.50%w. Disposal of cheese-whey is a critical issue associated with the dairy industries. The recent environment regulations have forced the cheese industry to allocate remarkable funds for the whey disposal. The capital appropriation to correspond the new regulations has caused the shutting down of several dairy

industries in the U.S. The new scenario has created the space for the development of alternative methods for the disposal cheese-whey. The presence of lactose at a remarkable concentration makes possible the use of whey as substrate for chemical bulk production by fermentation. The substrate would be available at very low price, or the fermentation process might be considered as a disposal process. A possible pretreatment of cheese-whey is the precipitation and recovery of the casein. The whey-filtrate free of casein is characterized by relatively low sugar content (4-5%w lactose).

The use of cheese-whey and lactose as substrates in ABE fermentation has been investigated by some researchers (Welsh and Veliky, 1984, 1986, Qureshi and Maddox, 1987). In terms of overall reactor productivities referred to batch fermentations, whey permeate resulted a relatively poor substrate when compared with starch and molasses substrates (solvents 5-15 g/L; productivity 0.1 g/Lh; yield0.23-0.41 $g_{solvents}/g_{substrate}$). Moreover, the concentration of total solvents reached at the end of the cheese-whey fermentations is lower than that reached at the end of the fermentation of conventional substrates. Therefore, the product (solvent) inhibition does not appear to control the performance of the fermentation. The butanol/acetone ratio at the end of the fermentation of whey by *C. acetobutylicum* is larger than that typically found during glucose fermentation. Linden et al. (1986) reported a butanol/acetone ratio as high as 20:1 for whey fermentation, well larger than that measured for glucose fermentation 3:1. The change of the pathway responsible of the butanol/acetone ratio increase is still not known. The studies available in literature just report a list of possible factors that may affect the butanol/acetone ratio. In particular, the temperature and the whey composition play a relevant role in the acetone and butanol production rate (Voget et al., 1989). The *C. acetobutylicum* P262 appears to be the most effective strain so far reported in the open literature for the production of solvents from whey permeate (Ennis and Maddox, 1985). Qureshi and Maddox (1987) studied the whey permeate conversion by means of *C. acetobutylicum* P262 in a biofilm reactor and reported a butanol production rate 16 times larger than that measured in batch cultures with free cells.

High sugar content beverages (HSCBs) - such as fruit juices, syrups, soft drinks, and sport drinks - may be a potential carbon source for the ABE fermentation. They contain sugars as sucrose, fructose, and glucose. Even though the fate of these beverages is to refresh people, a huge quantity of them are yearly disposed. As a matter of fact, the disposal of industrial wastewater streams, seizures of illegal goods, and expiry date beverages is a critical issue for the high carbon content. Dwidar et al. (2012) adopted carbonated beverages as carbon source to successfully produce bioethanol. The preliminary tests to produce butanol from a carbonated beverage were very promising but a thorough characterization of butanol production by adopting high sugar content beverages is still absent in the literature.

1.3.2 Reactor Configurations

Design and operation of bio-reactors play an key role in the biotechnological industry. The knowledge of the rate of reactions, transport phenomena, hydrodynamics, and operating conditions allows to improve reactor performances and productivities, and then the process economics (Maddox, 1989; Qureshi and Blaschek, 2001). In the last twenty years several reactor configurations have been investigated to enhance the low productivity typical of AB fermentation process, often without any reference to the classical engineering principles (Villadsen, 2007).

Several configurations of batch reactors have been studied. The main disadvantage of this reactor typology is the low productivity due to three issues:
a) the necessity to grow at each cycle the biomass load for the solvent production;
b) the low AB concentration reached at the end of the fermentation;
c) the long downtime characteristic of discontinuous fermentation.

Nevertheless, the large scale batch fermentation was commonly used at the beginning of the butanol production on because it was: i) typically adopted in fermentation processes; ii) the best condition known to contain the product toxicity associated with ABE fermentation. During the 1940s and 1950s, the biobutanol production on industrial scale was carried out in large batch fermentors ranging in capacity between 200 and 800 m^3. Of course, the ABE fermentation process can not be economically competitive if carried out according to traditional conditions.

The fed batch technique was also investigated (Tashiro et al., 2004; Ezeji et al., 2004b). Unfortunately, the results were not promising as regards the improvement in the productivity and solvent yield.

Continuous cultures are typically charcaterizaed by solvents concentration and yield as large as batch cultures. Moreover, they are often characterized by improved productivity. The main disadvantage of this operation modality is related to the process stability: although high solvent yields can be achieved for long operation time, the solvent production is not stable and reduces with time, with a concomitant increase in acid formation (Schuster et al., 2001).

Leung and Wang (1981) were successful in carrying out a *C. acetobutylicum ATCC* 824 fermentation on a glucose-limited (50 g/L) complex medium. They operated with free-cell reactor. At dilution rate of 0.1 h^{-1}, they obtained a production of 15.9 $g_{solvents}$/L, with a yield of 0.32 g/g and a productivity of 1.5 g/Lh. At dilution rate of 0.22 h^{-1}, a maximum productivity of 2.55 g/Lh was obtained, but the solvent yields and concentration reduced to 0.26 g/g and 12 g/L, respectively.

Two- or multi-stage continuous fermentation systems have been investigated since 1958 in an attempt to separate the growth phase (acidogenesis) from the production stage (solventogenesis). Dyr et al. (1958) utilized a series of five fermentors and a dilution rate of 0.3 h^{-1}. The cell growth was maximized in the first fermentor, acids production occurred in the first two fermentors, and neutral solvent production occurred in the last three fermentors. Nevertheless the complex system adopted, the final solvent concentration and ratio were the same as in batch culture.
Interesting results were obtained by Mutschlechner et al. (2000) in a two-stage continuous fermentation of *C. beijerinckii* NRRL B592 on glucose. The first fermentor was operated at a dilution rate of 0.12 h^{-1} and the second was operated at 2.2 $10^{-2}h^{-1}$.

Performances of the reactor system were: solvent concentrations of ~15 g/L (4.8, 9.1, and 0.9 g/L, acetone, butanol, and ethanol), solvent yield of 0.25 g/g, productivity of 0.27 g/Lh. The final solvent concentration was close to that obtained in batch fermentations under operating conditions emulating those adopted during the continuous operation. On the basis of the promising results, a pilot-plant has been developed in Austria (Nimcevic and Gapes, 2000).

Although the success in the butanol production, all reported studies regarding continuous free cells cultures are characterized by low specific productivity.

The scenario turned out from the results reported in literature regarding batch and continuous process adopting free-cells do not appear promising. The long downtime characteristic of the discontinuous fermentation may be overcome adopting continuous culture of *C. acetobutylicum*. However, the very low growth rate of cells under solventogenesis conditions imposes to operate the reactor at very low

dilution rate, and then at low productivity. The feasibility of the ABE fermentation requires inevitably the achieving of better levels of process intensification by bioreactor design. Process intensification, i.e. the adoption of strategies that enable larger throughput per unit volume of reactor, brings about reduction of capital and operating costs that may represent a prerequisite for successful implementation of the ABE production. With reference to microrganism-based processes, process intensification is usually achieved by:

• containment of the biocatalyst within the reactor, by means of semipermeable membranes;

• immobilizing the microbial cells on a support or by favouring the conditions that encourage self-agglomeration of microbial cells into pellets.

Membrane reactors are characterized by both the confinement of cells into the reactor and the control of the liquid-phase draining from the reactor. Under these conditions, cells concentration may be theoretically increased up to any values (Mehaia and Cheryan, 1984). The cell concentration in the reactor is the result of a delicate equilibrium between growth rate under solventogenic conditions and cell died rate. Moreover, rheological phenomena as well as transport phenomena may play a relevant role in the cell concentration value that may establish in the membrane reactor. Under these conditions, the specific productivities of metabolites (acids and solvents) may be tuned acting independently on the cell concentration in the reactor and on the reaction environment. Interesting results were obtained by Pierrot et al. (1986) and Afschar et al. (1985). Pierrot et al. (1986) carried out the fermentation of *C. acetobutylicum* ATCC 824 on glucose under partial cell recycling and at a dilution rate of 0.5 h^{-1}. During the test a steady state regime established for several days characterized by cellular concentration of 20 g/L, total solvent concentration of 13 g/L and a solvent productivity of 6.5 g/Lh. Nevertheless the promising results, the spread of this reactor at industrial scale level is still limited by the cost of the membrane that, among other things, suffers of biofouling.

Biofilm reactor is a technology adopted since the beginning of the last century in several biotechnological processes (Kolot, 1984). It has been particularly investigated as regard wastewater bioremediation, a process characterized by a low added value. Therefore, any clever device for an economic intensification is welcome.

The production of solvents coupled with the non-growing solventogenic phase observed in batch cultures suggests that immobilized cell systems may be more suited for solvent production than continuous cultures utilizing free cells. The advantages of immobilized cell systems include: i) the physical retention of the cells in the reactor, facilitating the separation of the cells from the products; ii) high cell concentrations, allowing smaller reactor volumes and greater productivity; iii) use of packed columns or fluidized-bed reactors; iii) the possibility of media containing just the minimum amount of species to support the cell growth under solventogenic conditions and the substrate to be converted into solvents. The main disadvantages of this reactor typology is basically related to the transport phenomena within the biofilm and to the gas phase produced during the bioconversion:

• Transport phenomena may reduce the solvents production rate of the immobilized cells: the inner regions of the biofilm are characterized by a substrate/products concentration lower/larger with respect to the bulk values and, therefore, the solvent production rate may be strongly reduced with respect to the corresponding to the bulk conditions.

• The gas (hydrogen and carbon dioxide) formed during the anaerobic fermentation may accumulates in the biofilm as microbubbles decreasing the bioparticle (biofilm

covered support) density. As a consequence the bioparticles may float and segregate with respect to the medium flow.

Results available in literature on the butanol productivities in biofilm reactors are very promising. Typically, the best production have been obtained by *C. acetobutylicum* P262 and *C. beijerinckii* BA101 immobilized onto bonechar and clay brick respectively, the best support investigated. Main results are about solvents productivities ranging between 6.5 (Qureshi and Maddox, 1988) to 15.8 g/Lh (Qureshi et al., 2000) for glucose as substrate and 4.5 g/Lh (Qureshi and Maddox, 1987) for lactose as substrate. Napoli et al. (2010) produced up to 4.5 g/Lh in a biofilm packed bed reactor fed with a lactose based medium.

1.4 Metabolism

1.4.1 Fermentative Metabolism of Clostridium Bacteria

The genus *Clostridium* is a heterogeneous collection of gram-positive, obligatory anaerobic, non-sulphate-reducing, spore-forming, rod-shaped bacteria (Montoya et al., 2001). Solventogenic *Clostridia* have received much attention in recent years, because of their ability to produce industrially relevant chemicals such as butanol and acetone. The *Clostridia* produce several enzymes that bring about the breakdown of polymeric carbohydrates into monomers (Fig.5). These enzymes include α-amylase, α-glycosidase, β-amylase, β-glucosidase, glucoamylase, pullulanase, and amylopullulanase (Ezeji et al., 2007a).

During the fermentation of *Clostridia*, two separate/sucessive phases occur: the exponential acidogenic phase and the solventogenic phase. Fermentation starts with the acidogenic phase is first with the Clostridia performing typical butyrate fermentation when growing on starch or sugars. The major products are butyrate (butyric acid), acetate (acetic acid), carbon dioxide, and hydrogen. Ethanol and acetone are formed at low concentration. The production of the acids results in the decresaes of pH which poses the threat of cell death. Imminent death is evaded by a major metabolic shift that takes place at the end of the exponential growth phase. This also marks the end of the acidogenic phase and the beginning of the solventogenic phase. The excreted acids are taken up to be converted into the neutral products, butanol and acetone (in a molar ratio of typically 2:1). Conversion of butyrate and acetate into solvents increases the pH again, which means the cells can be metabolically active for a longer time. However, the solvents may also kill the cells, with butanol being the most toxic. Solvents inactivate the membrane proteins and destroy the membranes of the cells. Therefore, there is a limitation to the maximum solvent concentration that can be achieved during fermentation, which is approximately 2 $\%_W$ (Dürre, 2008).

Under conditions of low initial substrate concentration in the medium and/or excess of nutrients, a state known as acid-crash can occur (Zverlov et al, 2006). Under these conditions the switch to the solventogenic phase does not work and the fermentation ends abruptly due to acid overproduction.

The solventogenic *Clostridia* have the benefit of producing a variety of fermentation products (acetone, butanol, ethanol, acetic acid, butyric acid, etc.). However, this spectrum can also be an undesirable property because the formation of unwanted byproducts results in a loss of available carbon (reduction of yield). Enzyme production and control of electron flow in the glycolytic pathway are very

important with regard to the regulation of the butanol fermentation pathways. Ferrodoxin is commonly present among the solventogenic *Clostridia*. A change in the type and quantity of fermentation products produced can be achieved with alteration in the direction of electron flow around reduced ferrodoxin (Ezeji et al., 2007b). Butanol yield should therefore respond to factors that influence the direction of electron flow and, since the electron flow can be reversed, researchers have tested the effect of numerous reducing compounds. Compounds tested include: carbon dioxide , methyl viologen, and neutral red. The presence of these electron carriers stimulates butanol and ethanol formation at the expense of acetone synthesis (Mitchell, 1998). Scientists still study the physiology of the bacterium and associated critical interactions between carbon pathways and electron flow. This research may lead to improved strains and to the development of an optimal fermentation medium.

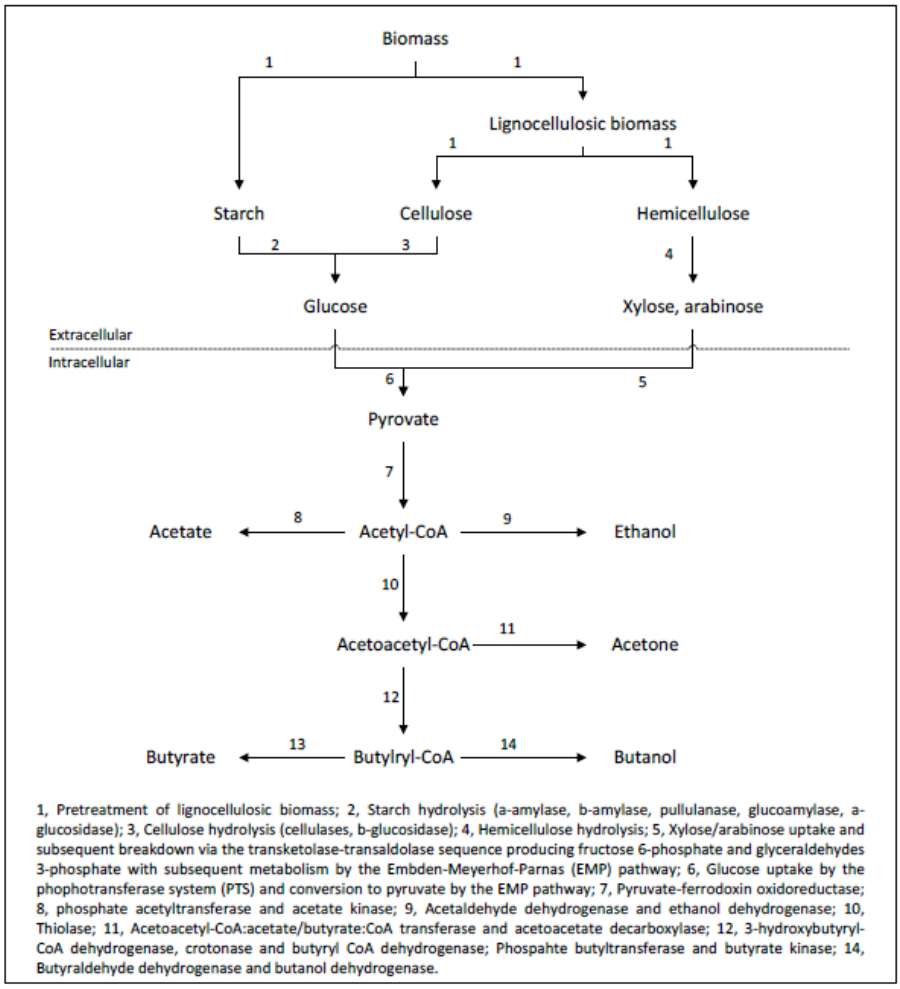

Fig.5: Simplified metabolism of biomass by solventogenic *Clostridia* (Ezeji et al, 2007b).

Strain development of industrial microorganisms has typically been performed through procedures of random mutagenesis and phenotypic selection. Metabolic engineering seeks to replace this 'shotgun' approach with a more rational effort (Bailey, 1991). The metabolic engineering approach is an iterative procedure involving identification of metabolic bottlenecks or limitations followed by manipulation to alter metabolic fluxes (Stephanopoulos and Sinskey, 1993). With the

advent of many powerful molecular biology techniques to manipulate the genetics of an organism, the emphasis has been on cloning and sequencing genes for enzymes associated with the desired product. The resultant genetic repertoire is a useful tool to manipulate the genetics of an organism to enhance product yields. However, the underlying assumption that product yields are only affected by the enzymes involved in the final steps of product synthesis is too simple. While levels of enzymes involved in the final steps of synthesis are important, significant enhancement of product yields may only be realized by taking a more global approach to cellular metabolism. A number of techniques have been developed to address the problem of understanding cellular metabolism. The initial efforts resulted in the development of metabolic control theory (MCA), in which information on enzyme kinetics is used to develop a global model of cellular metabolism (Kacser and Burns, 1973). Unfortunately, the lack of sufficient kinetic information renders MCA impractical for most biological systems. An alternative approach, metabolic flux analysis (MFA), using metabolic pathway balances to develop a model of cellular metabolism was reported by Papoutsakis (1984). This model takes the form of a system of linear equations based on species balances and in vivo metabolic pathway fluxes. The system of equations is typically underdetermined and must be further manipulated to be useful. Papoutsakis utilized biological constraints in the form of pseudo-steady state approximations on metabolic intermediates to reduce the underdetermined nature of the stoichiometric matrix. The reduced matrix was then used to develop a so-called 'fermentation equation' to verify fermentation data consistency, to develop 'gateway' sensors (which estimate unobservable physiological parameters from measurable quantities), and to predict maximum theoretical yields (Papoutsakis 1984). However, the presence of an unresolved singularity has prevented the calculation of some pathway fluxes critical in the metabolism of solventogenic clostridia. The determination of these unresolved fluxes responsible for acetate, butyrate, and acetone production is required to develop a better understanding of the metabolism of solventogenic clostridia.

1.4.2 Butanol Producing Clostridia

The yield of glucose to butanol is not as impressive as that of glucose to ethanol since butanol is normally produced together with acetone and ethanol in a ratio of 6:3:1. CO_2 and H_2 are major side products of the acid and solvent formation, and theis molar ratio is about 1.5:1. During fermentation approximately 3 moles of CO_2 and H_2 are formed per mole of hexose (glucose): 1.7 ton of gasses are formed per ton of solvents (97 wt% CO_2 and 3 wt% H_2) (Zverlov et al., 2006).

Table 2 shows stoichiometric equations for solvent production from glucose. Gapes (2000) estimated the theoretical limits of energy and mass yields if the ratio of the products, as stated above, is maintained. He adopted the data reported in Table 2, together with chemical properties and found: theoretical mass yield of 34%, and a theoretical energy yield of 94%. These limits and the maximum solvent concentration - approximately 2% - must be taken into consideration when comparing different strains. The first patented strains includes a few that were able to utilize between 4-6% fermentable sugars producing solvent concentrations of 14-18 g/L at solvent yields from 25-30%, under optimal conditions. Successive patented strains were able reported to utilize 7.5% fermentable sugars to give reproducible solvent concentrations of 18-23 g/L and yields of 30-33% (Walton and Martin, 1979; Shaheen et al., 2000).

Tab. 2. Stoichiometric Equations for Glucose Fermentation.

Product	Stoichiometric Equation
Acetone	$C_6H_{12}O_6 + H_2O \rightarrow C_3H_6O + 3CO_2 + 4H_2$
1-butanol	$C_6H_{12}O_6 \rightarrow C_4H_{10}O + 2CO_2 + H_2O$
Ethanol	$C_6H_{12}O_6 \rightarrow 2C_2H_6O + 2CO_2$
butyrate	$C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2CO_2 + 2H_2$
acetate	$C_6H_{12}O_6 \rightarrow 2C_2H_4O_2 + 2CO_2 + 4H_2$

Environmental factors as substrate medium composition and growth conditions can also greatly affect the composition of the fermentation end products (Montoya et al., 2001). On one hand, culture conditions utilized for comparative studies might be close to optimum for species and strains. On the other hand, it has to be accepted that it is unlikely that the specific conditions used would be optimal for all strains tested in that study. Indeed culture conditions vary from one study to another. Moreover, the upgrade to industrial-scale fermentations can not reproduce solvents levels comparable to those produced in laboratory-scale fermentations (Shaheen et al., 2000).

Main solvent-producing *Clostridia* may be classified into four groups: *C. acetobutylicum*, *C.beijerinckii*, *C.saccharobutylicum* and *C.saccharoperbutylacetonicum*. *C. acetobutylicum* (Fig.6) is a species that is phylogenetically distinct and very distantly related to the other three solvent-producing *Clostridia*.

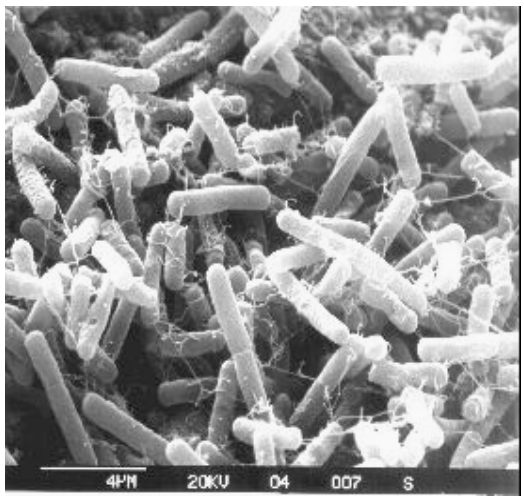

Fig.6: *Clostridium acetobutylicum* cells under the electron microscope [Princeton University, 2007].

This strain thrives on starch-based substrates and all the original starch-fermenting strains belong to this species (Shaheen et al., 2000). *C. acetobutylicum* is the best-studied solventogenic *clostridium* and many engineered strains have been developed of this species (Syed, 1994; Dürre, 1998; Shaheen et al., 2000; Dürre, 2008). Shaheen et al. (2000) carried out a comparative fermentation study on solvent producing *Clostridia* and they found that none of the three tested *C. acetobutylicum* strains performed well in either of the glucose or molasses media used. The highest solvents concentration was 9.5 g/L at 15.8% yield. However, these strains did perform better when they were tested in a maize medium.

C. beijerinckii is more related to *C. saccharobutylicum* and *C. saccharoperbutylacetonicum.* These three are known as the saccharolytic strains. The majority of these saccharolytic industrial strains belong to the *C. beijerinckii*

species. Although *C. acetobutylicum* is the best-studied solventogenic *clostridium*, it appears that *C. beijerinckii* might have greater potential for the industrial production of bio-solvents. *C. beijerinckii* has a wider optimum pH range for growth and solvent production, and the genetic potential to utilize a wider variety of carbohydrates (Ezeji et al., 2004a). Due to the location of the genes in *C. beijerinckii*, it is suggested that this strain is less susceptible to acid crash and therefore more suitable for longer (continuous) fermentations than *C. acetobutylicum* (Grube and Gapes, 2002). In the comparative study by Shaheen et al. (2000) the NCP 260 strain performed the best.

Ezeji et al. (2004a) did extensive studies on *C. beijerinckii* BA101, a mutated strain selected by mutagenesis of *C. beijerinckii* NCIMB 8052. This is a very versatile strain that performed well on a variety of substrates giving total ABE concentrations of 14.8-26.1g/L with yields of 37-50%.

C. saccharobutylicum and *C. saccharoperbutylacetonicum* are strains not very well charcaterized as regards fermentation studies. Shaheen et al. (2000) included these strains in a comparative fermentation study and they found that performance is better on glucose and molasses than on maize. The best fermentation result was obtained with the industrial strain *C. saccharobutylicum* BAS/B3/SW/336(S) by adopting molasses as substrate with a fermentable sugar concentration of 6.5%. The average solvent concentration was 19.6 g/L with a yield of 30%.

The ultimate goal is to generate strains with a competitive commercial position, which can be used in industrial biobutanol production. The above strains are almost all products of the traditional mutagenesis and selection techniques employed to improve the performance of solventogenic *Clostridia*. Further improvement can be made by modifying targeted metabolic pathways in the *Clostridia,* according to recombinant DNA technology. Although progress has been made, this technology has so far not yielded a hyper-butanol-producing industrial strain (Ezeji et al., 2007a). The analysis of the currently available *Clostridia* strains suggests that advanced fermentation and recovery techniques are the best short-term solutions to improve fermentative butanol production.

2 BUTANOL FERMENTATION AT UNIVERSITY OF NAPOLI

In 2007 at the University of Napoli started a research program aimed at the production of butanol by biotechnological route. An exhaustive overview of 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 and the yields of cell growth and metabolites produced during the lactose fermentation by *C. acetobutylicum*. The study regarded both experimental and theoretical aspects of the fermentation. The investigation was carried out under batch and continuous cultures of free *C. acetobutylicum* cells.

Continuous cultures were carried out under a wide interval of operating conditions in order to characterize the fermentation process under both the acidogenesis (Napoli et al., 2011) and the solventogenesis phases (Napoli et al., 2009). Accordingly, two typologies of reactors were designed, set-up and operated: an isotherm CSTR equipped with a pH controller, and an isotherm CSTR equipped with a pH controller and a microfiltration unit. Tests with the first reactor were designed for the characterization of the fermentation process under acidogenesis conditions. Tests with the second reactor were designed for the characterization of the solventogenesis phase.

The theoretical study was aimed at the assessment of a kinetic model and a yield model for cell growth and metabolites. The role of single metabolites and of the pH was investigated.

Napoli developed a first version of continuous biofilm reactor for the ABE production (Napoli et al., 2010). The study aimed at the optimization of the cells immobilization process and at the assessment of the butanol production by means of *C. acetobutylicum* biofilm. A biofilm fixed bed reactor was designed, set-up and successfully operated. The operating conditions of the continuous tests were selected to maximize the butanol production and butanol selectivity in agreement with results of the characterization of the process.

Tests suggest that the reactor performances are affected by the dilution rate and the pH. The maximum butanol productivity was the one of the largest values reported in the literature regarding the lactose conversion: 4.43 g/Lh. The butanol selectivity with respect solvents (88%W) was among the highest value reported in the literature.

3 AIM OF THE THESIS

The study carried out during the present Ph.D. program aimed at investigating the Acetone-Butanol-Ethanol (ABE) production process by fermentation. The work was carried out at the Dipartimento di Ingenria Chimica, dei Materiale e della Produzione Industriale of the Università degli Studi di Napoli 'Federico II'. The activities were articulated along three lines: i) the characterization of the ABE fermentation process as regards kinetics and yields using different renewable resources (lignocellulosic biomass, high sugar content beverges and cheese whey); ii) the characterization of the ABE fermentation process according to the metabolic flux analaysis (MFA) and to the dynamic kinetic models; iii) the development of innovative continuous biofilm reactor for the ABE production.

A commercial clostridia strain was investigated. *Clostridium acetobutylicum* DSM 792 was selected for its ability to produce ABE with satisfactory selectivity towards the butanol.

The ABE fermentation process by adopting renewable resources. Characterization in terms of kinetics and yields.

The study was aimed at the assessment of both the kinetics and the yields of cell growth and metabolites produced during the fermentation of:

- simple sugars (glucose, mannose, arabinose and xylose) representative of lignocellulosic biomass hydrolyis;
- simple sugars (glucose, fructose and sucrose) typically present in high sugar content beverages;
- high sugar content beverages.

The investigation was carried out under batch conditions with free *C.acetobutyicum* cells. Batch tests were focused on the preliminary characterization of the fermentation with the aim of highlighting the relevant features of the process. The process has been characterized in terms of kinetics and yields under a wide interval of operating conditions (substrate concentration, nutrient concentrations, …).

Characterization of the ABE fermentation process. MFA and dynamic kinetic models.

The MFA and the kinetic dynamic methodology were adopted to characterize the ABE fermentation. Selected batch fermentation tests were carried out with the aim to charcaterize the time-evolution of the concentration of substrate, cells and metabolities. Tests were carried out at initial sugar concentration 60 g/L. The time-series of concentration were processed according the MFA and the kinetic model methodology. In particular:

- the MFA was adopted to investigate the role of the main reaction steps of the *Clostridium acetobutylicum* metabolic pathway to convert reference sugars (glucose and xylose) of hydrolyzed lignocellulosic biomass into butanol. Results of batch fermentation tests carried out using glucose and xylose as carbon source were adopted for the flux assessment. The stoichiometric matrix of the model was characterized by a singularity that prevented the assessment of a unique set of fluxes of the primary metabolic activity. The non linear constrain proposed by Desai et al. (1999) relating the acetate and butyrate uptake fluxes was adopted to solve the model equation set. The MFA was proposed with reference to glucose and xylose as carbon source. The

comparison of the assessed fluxes suggested the role of each reaction step as a function of the carbon source investigated.

- A kinetic dynamic model of acetone–butanol–ethanol (ABE) production by *Clostridium acetobutylicum* DSM 792 was proposed using the biochemical networks simulator COPASI. Effects of substrate were studied implementing the model with different sugars: glucose, mannose, fructose, sucrose, lactose, xylose and arabinose. If necessary, the metabolic pathway was modified according to the specific sugar. In particular, the Embden-Meyerhof-Parnas (EMP) pathway equations were used for hexose and disaccharide sugars while the pentose phosphate (PP) pathway equations were used for pentose sugars.

Development of innovative continuous biofilm reactor for the ABE production.

The study was aimed at the assessment of the butanol production by means of *C. acetobutylicum* biofilm reactor. The activity aimed at the butanol production regarded the design, set-up and operation of a biofilm fixed bed reactor. Unsupplemented cheese whey was adopted as renewable feedstock. Operating conditions of the continuous tests were selected to maximize the butanol production and butanol selectivity. The activity included:

- The selection of a pre-treatment procedure of the cheese whey. The process was coupled with fermentation tests carried out under batch conditions to assess effects of the feedstock pre-treatment on fermentation performances;
- Tests with the pre-treated cheese whey to characterize the fermentation process in terms of butanol production and butanol selectivity;
- Design, set-up and optimization of a fixed bed biofilm reactor for cheese whey conversion;
- Tests with the biofilm reactor under continuous conditions.

The activity was completed with the development of an innovative continuous biofilm reactor configuration: “carosello” of packed bed biofilm reactors connected in series. The reactor system has been equipped with a device to switch the feeding at the reactor according to a pre-set sequence. A mathematical model to support the bioreactor system design was developed.

The thesis separately addresses the three investigated lines. Section 4 regards the fermentation characterization of lignocellulose representative sugars and of high sugar content beverages. Section 5 reports on the MFA and dynamic kinetic models adopted for the characterization of the ABE fermentation process. Section 6 addresses the butanol productions in a biofilm reactor and the development of an innovative continuous biofilm reactor configuration. Within each section manuscripts submitted to scientific journals and pertinent to the topic are included.

4 ALTERNATIVE RENEWABLE FEEDSTOCKS

The activity focused on two typologies of feedstocks: lignocellulosic biomass and high sugar content beverages. The attention for these two categories has been highlighted in the introduction sections. As a summary, the lignocellulosic biomass is a huge renewable resources belonging to the second generation feedstocks. It may be produced or may be harvetsed as waste material in several production processes. The sources of high sugars content beverages are manifold: waste material of beverage industries, seizures of illegal goods, and expiry date beverages. They are a critical issue for the disposal but may be considered a good feedstocks.

The section 4.1 reports results of tests carried out with sugras representative of the hydrolysis of lignocellulosic biomass. Main results of tests carried out with mixtures of sugars are reported in section 4.2. Results are promising and further research should be carried out to charcaterize the fermentation of these systems from the kinetics point of view. The section 4.3 reports results of tests carried out with high sugars content beverages.

4.1 Butanol Production From Lignocellulosic-Based Hexose And Pentoses By Fermentation Of Clostridium Acetobutylicum

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ABSTRACT

The characterization of the ABE fermentation by *C. acetobutylicum* DSM 792 is reported, adopting sugars representative for hydrolysis products of lignocellulosic biomass: glucose, mannose, arabinose and xylose. Batch fermentations of single sugars were carried out changing the concentration of each nutritional factors used for the preparation of the synthetic medium.

C. acetobutylicum was able to grow on the investigated sugars and to produce ABE. The optimal conditions for the fermentation were critically assessed taking into account the possible co-fermentation of the investigated sugars and looking at the maximum exploitation of substrates. In particular, the optimal medium depended on the investigated sugar and differed from that typically suggested for glucose fermentation to produce butanol. As a consequence, optimisation of medium for a mixture of sugars will be necessary and the composition depends on the aim of the process: ABE maximization vs. substrate conversion.

Keywords: bioreactors, *Clostridium acetobutylicum*, batch processing, optimization, pentoses

INTRODUCTION

The continuous depletion of worldwide oil deposits coupled with the awareness of the modern society about the concentration increase of greenhouse gas – emitted during combustion of fossil fuels - has recently revived the interest in the production

of biofuel. Both industrial and academic researches have paid attention to the development of (bio)sustainable processes and to the adoption of renewable feedstocks. The spectrum of biofuels includes butanol, a simple four carbon alcohol characterized by interesting features (Cascone, 2008; Durre, 2007; Saravanan et al., 2010): low vapour pressure, blending with either gasoline or diesel at any fraction, energy content close to that of gasoline, compatible with current configuration of engines without any retrofitting. Butanol may be produced from renewable resources (biomass) by the Acetone-Butanol-Ethanol (ABE) fermentation process.

ABE is typically produced during the later stage of batch fermentations of some Clostridium strains - saccharolytic butyric acid-producing bacteria - under appropriate operating conditions (*Clostridium saccharoperbutylacetonicum*, *C. acetobutylicum*, *C. beijerinckii*, *C. aurantibutyricum*). These strains are able to metabolize a great variety of substrates, pentoses, hexoses, mono-, di- and polysaccharides (Tashiro et al., 2004). Under batch conditions the fermentation process of solvent-producing clostridium strains proceeds with the production of cells, hydrogen, carbon dioxide, acetic acid and butyric acid during the initial growth phase (acidogenesis) (Jones and Woods, 1986). As the acid concentrations increase (pH decrease), the metabolism of cells shifts to solvent production (solventogenesis) and acidogenic cells – able to reproduce themselves - shift to the solventogenesis state with a morphological change (Jones and Woods, 1986). During solventogenesis the active cells become endospores unable to reproduce themselves. Two different physiological states must be taken into account for *Clostridia*: one for the acidogenic phase, and one for the solventogenic phase.

Major challenges with ABE fermentation concern four issues: i) the butanol toxicity to microbial cells; ii) the butanol productivity; iii) the cost of fermentation feedstocks; and iv) the cost of the butanol recovery from the fermentation broth (Napoli et al., 2012). Regarding butanol toxicity on cells, recent developments of molecular techniques applied to solventogenic microorganisms in combination with advances in fermentation technology and downstream processing have contributed to improve feasibility and competitiveness of the ABE fermentation process. The ABE concentration and productivity in batch fermentations by solventogenic *Clostridium* species can be increased to values between 13 to 18 g/L and 0.2 to 0.3 g/L/h, respectively (Ezeji et al., 2008). Modest increases in ABE titer and productivity can be achieved by supplementing acetate (Chen and Blaschek, 1999) or butanol (Junne et al., 2006) to the fermentation medium.

Fermentation substrate is an important factor affecting the cost of butanol production (Kumar and K. Gayen, 2011; Qureshi and Blaschek, 2000). Although a potential advantage comes from adopting waste materials as feedstocks (Ezeji and Blaschek, 2008; Ezeji et al., 2007b; Liu et al., 2010; Napoli et al., 2010), their supply may fluctuate over time and it may not be sufficient to fulfil biofuel demand. Lignocellulose is the most abundant renewable resource on the planet, it fits as feedstock of biorefineries (Friedl, 2012) and it has great potential as a substrate for fermentation because of the un-competitiveness with food resources (Ezeji and Blaschek, 2010; Jurges et al., 2012). Despite the advantages in sustainability and availability, the commercial use of lignocellulose is still problematic. Due to the complexity of lignocellulosic materials, hydrolysis of hemicellulose and cellulose into five- and six-carbon sugars has to be carried out prior to, or concurrently with, the fermentation (Lee et al., 1996). Some reports on the corresponding ability of Clostridia strains are available in the scientific literature, but no systematic investigation has been carried out (Jurges et al., 2012).

The present study reports about a research carried out at the Department of Chemical Engineering of the University of Napoli Federico II on the ABE fermentation by *Clostridium acetobutylicum* DSM 792, adopting feedstocks from lignocellulosic biomass. The activity was focused on fermentation of sugars representative for hydrolysis products of lignocellulosic biomass: hexoses (glucose and mannose), and pentoses (arabinose and xylose). Batch fermentations of single sugars were also aimed to assess the effects of the medium composition: the concentration of K_2HPO_4/KH_2PO_4, $FeSO_4$, $MnSO_4$, and $MgSO_4$ were changed in wide intervals. Tests were also carried out supplementing the medium with $CaCO_3$. The conversion process was characterized as a function of time in terms of biomass, acids and solvents concentrations as well as pH and total organic compounds.

MATERIALS AND METHODS

Microorganism and media

Clostridium acetobutylicum DSM 792 was supplied by DSMZ (Braunschweig, Germany). Stock cultures were reactivated according to the DSMZ procedure. Reactivated cultures were stored at -80°C. The thawed cells were inoculated into 12 mL synthetic medium containing glucose (30 g/L) and Yeast Extract (5 g/L) in 15 mL Hungate tubes (pre-cultures). Cells were grown under anaerobic conditions for 48 h at 37 °C, then they were transferred into fermentation bottles; in order to ensure reproducibility each test was done in duplicate, the reported results are the mean values between them.

The standard fermentation medium consisted of 5 g/L YE and 2 g/L ammonium chloride (Nitrogen Source) supplemented to P2 stock solution: buffer) 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4; mineral) 0.2 g/L $MgSO_4.7H_2O$, 0.01 g/L $MnSO_4.7H_2O$, 0.01 g/L $FeSO_4.7H_2O$ [11]. The medium was sterilized in autoclave prior to the carbon source addition. Chemicals ($CaCO_3$, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $MnSO_4.7H_2O$, $FeSO_4.7H_2O$) and yeast extract were from Sigma Aldrich.

Four sugars were investigated: glucose, mannose, arabinose, and xylose (Sigma Aldrich). Concentrated solutions of sugars were sterilized by filtration and supplemented to the autoclaved medium. The stock solution concentration of each investigated sugar was: glucose 300 g/L, mannose 300 g/L, arabinose 300 g/L, and xylose 300 g/L.

Analytical methods

pH was measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments). The analysis of culture samples withdrawn from cultures provided concentration in the liquid phase for biomass, sugars and metabolites, total organic carbon (TOC), and total nitrogen (TN). 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 OD_{600} = 0.4 $_{gDM}$/L (Napoli et al., 2011).

The concentration of soluble species was measured in the liquid phase after cell harvesting by centrifugation. Sugar concentration was measured by high performance liquid chromatography (HPLC) using an Agilent 1100 system (Palo Alto, CA). 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. Deionized water was used as mobile phase at a flow rate of 0.6 mL/min. Metabolite concentrations were measured by means of a GC apparatus equipped with a FID, and outfitted with a capillary column poraplot Q (25 m x 0.32 mm) was used. Internal standard (hexanoic

acid) was adopted to assess acids and alcohols and their concentrations. The TOC/TN was measured with a Shimadzu TOC 5000A analyzer.

Operating conditions and procedures

Pyrex screw capped bottles (100 mL) containing 75 mL medium were used as fermenters. All experiments were carried out in fermenters at rest, at 37 °C, without pH control. The medium was inoculated with 6.25 % (v/v) suspension of active growing pre-cultures. 3 mL of cultures were sampled periodically for cell/metabolites characterization.

The initial concentration of the investigated sugar in each batch was set at 60 g/L. Table 1 reports the interval of concentration of the nutritional factors investigated.

Nutritional Factors	Concentrations (g/L)
KH_2PO_4 - K_2HPO_4	0-5
$CaCO_3$	0-10
$FeSO_4$ $7H_2O$	0.002-0.01
$MgSO_4 7H_2O$	0-2
$MnSO_4 7H_2O$	0-0.1

Table 1. Composition of the medium investigated.

Batch fermentations were characterized in terms of cell growth, pH, sugar conversion, and acids and solvents production. Measurements were worked out to assess the following data.

- overall sugar conversion (ξ_S), i.e. the ratio between the sugar converted and the initial sugar $(S_0 - S)/S_0$
- sugar-to-product"i" yield coefficient ($Y_{i/S}$), i.e., the ratio between the produced mass of product "i" (cells or butanol) and the related decrease of the substrate mass.
- the initial specific sugar consumption rate (r_S^0). It was estimated at the beginning of the exponential phase as the slope of the sugar concentration (S) vs. time curve, divided by the biomass concentration measured at the exponential phase onset.
- the maximum specific butanol production rate (r_B^{MAX}). It was estimated at the onset of the solventogenic phase as the slope of the butanol concentration (C_B) vs. time curve, divided by the cells concentration measured at the solventogenesis onset.
- maximum butanol, ABE and cell concentrations. The maximum value of concentration measured during the batch fermentation was reported.
- residual acids concentration. The concentration of acids at the end of the solventogenic phase.

RESULTS AND DISCUSSION

Sugar effects. Batch fermentation tests were carried out in the standard medium adopting as carbon source the investigated sugars: glucose, mannose, arabinose, and xylose.

Fig.1 reports the time resolved profiles of pH and of the concentration of *C. acetobutylicum* cells (X), xylose, and metabolites (acetic acid, butyric acid, ethanol, acetone and butanol) measured during a batch culture. The analysis of the data confirmed the typical two-phase behaviour of the fermentation (Jones and Woods, 1986): acidogenic phase and solventogenic phase.

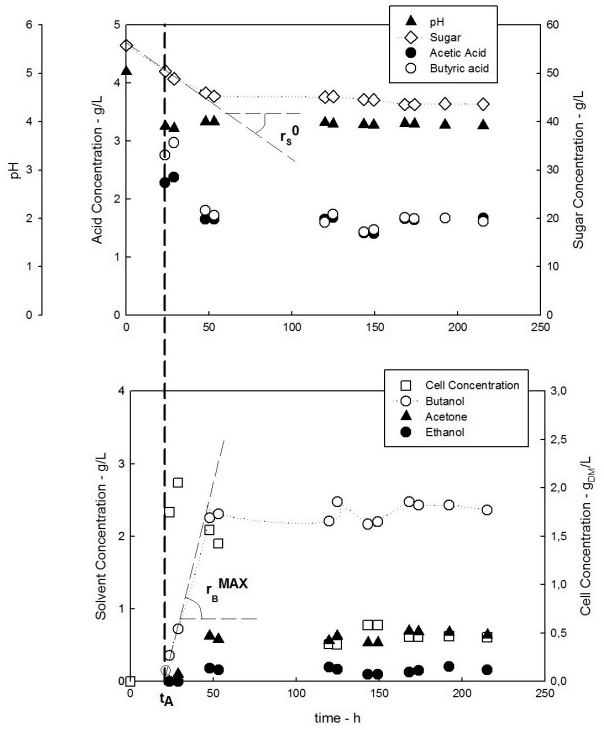

Fig. 1. Data measured during *C. acetobutylicum* fermentation in standard medium supplemented with xylose (nominal initial concentration: 60 g/L). The vertical dashed line marks the beginning of the solventogenesis phase

After a lag phase of about 10 hours, the acidogenic phase was characterized by: i) continuous conversion of the substrate; ii) steady increase of concentration of cells and acids; iii) pH decrease. As the pH approached 4 (t_A=22 h), the solventogenic phase was initiated. The solventogenic phase is characterized by: i) gradual decrease of the sugar concentration approaching a stationary value; ii) gradual decrease of the cell concentration as a consequence of cell lysis (Ezeji et al., 2007c); iii) steady increase of concentration of solvents approaching a stationary value; iv) gradual decrease of the acids concentration as a consequence of their conversion by clostridium cells at a rate larger than their production.

Fig.2 reports the time-series of both sugar concentration and solvent (acetone + butanol + ethanol) concentration measured during the batch fermentations carried out with the standard medium supplemented with the investigated sugars. The analysis of Fig.2 and of data reported in Table 2 points out the issues reported hereinafter.

C. acetobutylicum was able to grow on the investigated sugars. According to previous investigations (Ezeji and Blaschek, 2008), the conversion degree decreased with the sugar species with the order glucose, arabinose, mannose, xylose. Sugars were converted into ABE. The fermentation performances expressed in terms of production rate and production yield of solvents depended on the sugar. Tests carried out with glucose confirmed the high performance typically reported for this sugar: 12.8 g/L ABE produced within 160 h, $Y_{X/S}$=0.11, $Y_{B/S}$=0.26; x_S=0.52. The acidogenesis phase lasted less than 1 day. The Acetone:Butanol:Ethanol molar ratio was 3:6:1. The residual acid concentration at the end of fermentation was 1.13 g/L. Except for the xylose, the fermentation performances assessed for tests carried out adopting the investigated sugars were practically similar to those assessed for tests carried out adopting glucose. The xylose fermentation was characterized by a high $Y_{X/S}$: 0.17 g/g.

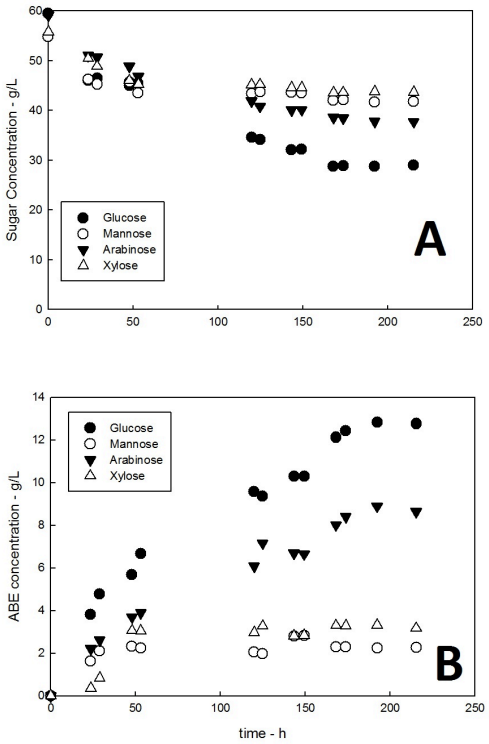

Fig. 2. Data measured during batch fermentations of *C. acetobutylicum* in standard medium supplemented with the investigated sugars Nominal initial concentration of the investigated sugar: 60 g/L. A) Residual sugar concentration. B) Total solvent (ABE) concentration.

The ABE production depends on the sugar with the following features: i) the acidogenesis phase time (t_A); ii) the solvent production time (defined as the time interval during which solvents accumulate in the broth); iii) the final concentration of ABE. t_A decreased with the order xylose, mannose, arabinose, and glucose. The two last features decreased with the sugar species with the order glucose, arabinose, mannose and xylose.

The residual acid concentration and the ABE yield depended on sugars (table 2). It is interesting to note that tests carried out with mannose and xylose are characterized by both high residual acid concentration and low ABE yield. It appears that the capacity of *C. acetobutylicum* to convert acids into ABE is low when mannose and xylose are adopted as carbon source compared to glucose and arabinose.

The effects of the medium composition on the fermentation performance with reference to each investigated sugar are hereinafter reported and discussed. The strategy was to change the composition of one medium component, to assess the optimal concentration of the selected component. Then successive test series were carried out, adopting the optimized concentration of the investigated components.

Buffer (KH_2PO_4 - K_2HPO_4). Table 1 reports data regarding fermentation tests carried out adopting the standard medium except for the buffer (KH_2PO_4 - K_2HPO_4) concentration. For each sugar investigated the buffer concentration changed between 0 and 5 g/L, mass ratio KH_2PO_4-K_2HPO_4 set at 1:1.

Tests carried out without buffer were characterized by absence of fermentation. Except for xylose, optimal fermentation performance - cell growth, solvent production and yields – were assessed for tests carried out at buffer concentration ranging between 0.25 and 0.5 g/L. The performances were practically constant within this interval for tests carried out with glucose, arabinose and xylose fermentations, they decrease with buffer concentration for tests carried out with mannose. The

performances of *C. acetobutylicum* fermentations adopting xylose as carbon source did not changeremarkably with the buffer concentration regarding butanol and total solvent concentrations and r_B^{MAX}. At 5 g/L buffer concentration, performances definitively decreased. It appeared that at high buffer concentration (5 g/L) - associated with high initial pH (data not reported) - enhanced the acidogenic phase and inhibited the solventogenic phase. As a result, the initial sugar specific consumption rate (r_S^0) increased with the buffer concentration. On the other hand, the solventogenic phases - butanol and total solvent concentrations and r_B^{MAX} – decreased at high buffer concentration.

The reported results suggest that the optimum buffer concentration should be 0.25 g/L when the four sugars are combined in the medium.

$CaCO_3$. Fig.3 shows time-resolved concentration of both sugar and total solvents measured during fermentation tests carried out adopting medium supplemented with $CaCO_3$ at 5 g/L.

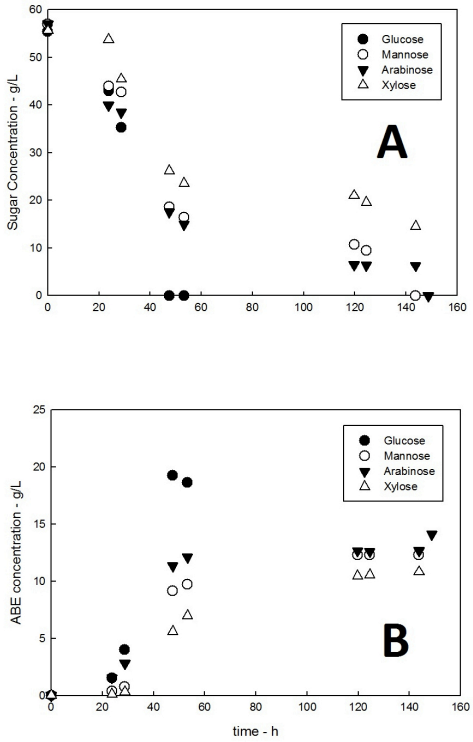

Fig. 3. Time resolved profiles of residual sugars (A) and ABE (B) concentrations measured during *C. acetobutylicum* fermentation tests. Standard medium: except buffer concentration set at 0.25 g/L. Initial $CaCO_3$ concentration: 5 g/L.

Table 3 summarizes and evaluates the data reported in Fig.3 and the fermentation tests carried out setting the $CaCO_3$ initial concentration at 0.1, 0.5, 5 and 10 g/L. The analysis of the effects of CaCO3 is reported hereinafter, taking also into account results reported in Table 2 (fermentation without $CaCO_3$).The comparison of Fig.2 and Fig.3 points out that fermentations carried out supplementing $CaCO_3$ are typically characterized by enhanced conversion - rate and degree - of the sugars, higher residual concentration of acids, and faster production of solvents. The maximum acids concentration increased with the initial $CaCO_3$ concentration. Nevertheless, the pH drop measured during acidogenesis did not change with initial $CaCO_3$ concentration (data not reported).

The measured high substrate conversion degree turned out in high total solvents and butanol concentration, while produced biomass did not increase remarkably, except for xylose. The ABE/biomass ratio vs. $CaCO_3$ concentration was

characterized by a maximum. The butanol/ABE ratio increased with $CaCO_3$ for tests carried out with hexoses - from about 2 without $CaCO_3$ up to about 3.5 at 10 g/L $CaCO_3$ and was approximately constant for tests carried out supplementing pentoses.

Fig.4 reports data of tests aimed at investigating the role of both substrate depletion and butanol inhibition on the *C. acetobutylicum* fermentation. Tests refer to operating conditions reported in table 3 characterized by substrate utilization: glucose, mannose, and arabinose supplemented with 5 g/L $CaCO_3$. The investigated sugar concentration was set at the initial nominal value (60 g/L) as the sugar conversion was about as low as 70-80% by supplementing a high concentrated sugar solution.

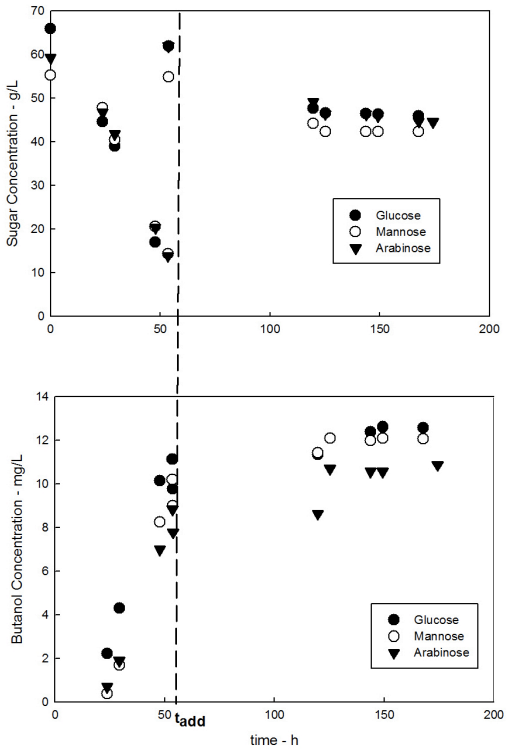

Fig. 4. Time resolved profiles of sugar concentration and butanol concentration. Medium: 0.25 g/L buffer, 5 g/L $CaCO_3$. Initial glucose concentration 60 g/L. At t_{add} glucose concentration was increased up to 60 g/L.

Fig.4 shows that sugars were successfully converted and butanol concentration approached a constant value depending on the sugar. The analysis of data suggests that under operating conditions tested: i) the *C. acetobutylicum* fermentation was inhibited by butanol, and ii) the butanol inhibition concentration decreased with the order glucose/mannose (13 g/L), arabinose (10.5 g/L), and xylose (8.5 g/L).

The reported results suggest that $CaCO_3$ promotes the butanol production, improves conditions for butanol recovery, and allows feedstock utilization. All these issues improve the butanol productivity competitiveness. Both the cost of feedstocks and of the butanol recovery system are the main items of the economic assessment of the ABE production (Napoli et al., 2012a; Qureshi and H.P. Blaschek, 2000). Regarding the promoting effects of $CaCO_3$ on butanol production, it may be interpreted by the increase of stability of membrane proteins, provided the presence of bivalent ions (Ca^{2+}) (Ezeji, 2011).

The analysis of the performances assessed with the four investigated sugars expressed in terms of maximum butanol concentration, ABE/biomass ratio, and

butanol/ABE ratio suggested that the optimal $CaCO_3$ concentration was 5 g/L. Therefore, the successive results regarding nitrogen, iron, magnesium, and manganese are reported with reference to tests carried out adopting a medium characterized by 0.25 g/L buffer concentration and 5 g/L $CaCO_3$ concentration.

Nitrogen. Tests regarding the effects of the nitrogen source on the performances of *C. acetobutylicum* fermentation were carried out setting buffer concentration at 0.25 g/L, $CaCO_3$ concentration at 5 g/L and adopting four nitrogen source systems characterized by constant nitrogen content (1.08 gN/L). The nitrogen source systems investigated were: i) YE, ii) YE and NH_4Cl, iii) NH_4Cl, and iv) $NaNO_3$.

No fermentation was observed in tests carried out adopting either NH_4Cl or $NaNO_3$ as nitrogen source. Whatever the sugar, the presence of YE resulted indispensable to *C. acetobutylicum* fermentation. However, the comparison of performances assessed during tests carried out supplementing either YE or YE and NH_4Cl showed (data not reported) that the absence of NH_4Cl improved the butanol productivity: without NH_4Cl the conversion time increased (the lag phase time increased) and the maximum butanol concentration was reduced (<5g/L).

According to the reported results, the successive investigation regarding iron, magnesium, and manganese are focused on tests carried out with a medium characterized by 0.25 g/L buffer concentration, 5 g/L $CaCO_3$ concentration, and YE and NH_4Cl solution.

$FeSO_4$. Two supplementary series of test were carried out to complete the comparison framework for the iron effects on *C. acetobutytlicum* fermentation. The supplementary tests were carried out at iron concentration of 0.002 g/L with and without $CaCO_3$ supplement (Table 4).

The analysis of data suggests that iron concentration affected mainly performances of fermentations carried out adopting hexoses as carbon source. On one hand, residual sugar concentration, butanol-to-sugar yield and biomass-to-sugar yield decreased with iron concentration. On the other hand, butanol to acetone ratio increased under iron limitation conditions.

Performance of fermentations carried out adopting pentoses as carbon source did not change with iron concentration.

The enhancement of the butanol to acetone molar ratio observed for tests carried out with hexoses may be interpreted taking into account the electron flow associated to the sugar conversion. The first steps of the glucose conversion pathway are characterized by electron flow directed to hydrogen production and carbon flow to acid biosynthesis (acetic and butyric acids). On one hand, the breakdown of glucose to acetyl coenzyme A provides reducing equivalents to cells. Indeed, Bahl et al. (1986) reported that “to recycle NAD+, NADH is oxidized by NADH ferredoxin reductase, while hydrogenase oxidizes reduced ferredoxine to produce molecular hydrogen”. On the other hand, during the solventogenic phase, both electron and carbon flux are directed to solvent production (butanol, acetone, and ethanol) via ferredoxine NAD(P)+ reductase (Peguin et al., 1994), and aldehyde and alcohol dehydrogenases. Moreover, it has been known that alcohol formation increased specifically by decreasing the in vivo activity of the hydrogenase of *C. acetobutylicum*. In particular, the in vivo activity of hydrogenase has been decreased by: i) increasing hydrogen partial pressure (Doremus et al., 1985); ii) flushing carbon monoxide (a reversible inhibitor of hydrogenase) (Meyer et al., 1986); iii) iron limitation (Bahl et al., 1986); iv) supplementing an artificial electron carrier such as methyl viologen (Kim et al., 1988) or neutral red (Hongo, 1958). In the investigated case, the decrease of iron concentration in the hexoses fermentation broth resulted

into a decreased hydrogenase activity and increased acohol formation, then an increased butanol/acetone ratio.

Although the beneficial effect of butanol selectivity observed during the hexoses fermentations under iron limitation, the attention was focused on standard iron concentration because it is characterized by higher productivity of solvents. Therefore, the successive sections regarding magnesium and manganese effects report tests carried out adopting a medium characterized by 0.25 g/L buffer concentration, 5 g/L $CaCO_3$ concentration, and standard concentration regarding nitrogen and iron sources.

$MgSO_4$. Table 5 reports all the relevant data regarding fermentation tests carried out supplementing media with $MgSO_4$ at concentrations ranging between 0 and 2 g/L, 0.2 g/L being the concentration in the standard medium. The analysis of data in table 5 points out the presence of a dual effect when Mg concentration changed:

- 0.2 g/L resulted an optimized concentration regarding sugar conversion, initial substrate uptake rate, and maximum biomass concentration;
- absence of $MgSO_4$ supported the butanol production (maximum butanol concentration and butanol-to-substrate yield) even though the maximum butanol production rate was not as high as measured under standard medium conditions.

As a consequence from the reported results, the optimal $MgSO_4$ concentration was set to the standard value: 0.2 g/L. For the hexoses, the decrease of the maximum butanol concentration is compensated by the increase of production rate. However, the operating value should be finely tuned in a continuous bioreactor since remarkable advantages may come form the reduction of $MgSO_4$ concentration: reduction of running cost of the process and enhancement of butanol production by pentoses conversion.

According to the above reported results, the analysis of the effects of $MnSO_4$ concentration on fermentation was focused on tests carried out adopting media with 0.2 g/L $MgSO_4$.

$MnSO_4$. Table 6 reports main results of tests aimed at investigating the effects of $MnSO_4$ concentration on fermentations. The composition of the medium was the standard except 0.25 g/L of buffer, 5 g/L of $CaCO_3$. The $MnSO_4$ concentration was increased up to 0.1 g/L.

The standard concentration of $MnSO_4$ confirmed to be the optimal for the glucose fermentation. It was not optimal for the other sugars. The performances of fermentations carried out supplementing mannose, arabinose, and xylose typically increased with the concentration of $MnSO_4$.

As a consequence of the reported results, the optimal $MnSO_4$ concentration may be set between 0.01 and 0.1 g/L when all the investigated sugars must be considered in a mixture. As a matter of fact, at a concentration higher than the standard value (0.01 g/L) the performance loss regarding the glucose conversion may be compensated by the enhancement of performances from the mannose, arabinose, and xylose conversion.

Main results were:

- *C. acetobutylicum* is capable to convert products of lignocellulosic biomass hydrolysates into solvents (ABE);
- The optimum buffer concentration was 0.25 g/L;
- $CaCO_3$ supplementation enhances the conversion degree of the substrate and the final solvent concentration, 5 g/L being the optimum concentration;

- The sugar to butanol yield and the butanol to acetone molar ratio increased in iron limitation conditions for glucose and mannose fermentations;
- Except for glucose, fermentation performances – maximum butanol production rate and maximum butanol concentration - increased with Mn_{2+} concentration, within the interval investigated. For tests carried out with glucose, fermentation performances were characterizes by maximum at MnSO4 0.01 g/L;
- Mg^{2+} positively affected glucose bioconversion. Fermentation performances were optimized at 0.2 g/L.

In conclusion, the analysis of results of all the batch fermentations carried out adopting sugars representative of hydrolysate of lignocellulosic biomass as carbon source pointed out that the ABE production optimisation needs an accurate selection of the medium. The composition typically adopted for glucose was not optimal for all sugars. Productivity and butanol selectivity could be optimised by fine tuning of the medium composition taking into account reported results. Moreover, fermentation performances – maximum butanol production rate and maximum butanol concentration - may be enhanced by supplementing the medium with $CaCO_3$.

Reported results will be integrated with kinetic characterization – specific growth rate and butanol production rate - of the bioconversion process, taking also into account sugar mixtures. Continuous tests will be also carried out for the assessment of kinetics and yields (Napoli et al., 2012b; Napoli et al., 2009; Raganati et al., 2013) and of enhancement effects of metabolites supplement to feedstocks (Junne et al., 2006).

CONCLUSIONS

Fermentation tests of *Clostridium acetobutylicum* adopting lignocellulose reference sugars (glucose, mannose, arabinose and xylose) were successful. Tests were aimed at investigating the optimal medium for butanol production. The composition of the medium – species and concentration – was changed in a wide interval of operating conditions.

The analysis of the results pointed out that the ABE production optimisation needs an accurate selection of the medium. The composition typically adopted for glucose was not optimal for all sugars. Productivity and butanol selectivity could be optimised by fine tuning of the medium composition taking into account reported results.

NOMENCLATURE

	Definitions	**Dimensions**
ABE	acetone-butanol-ethanol	-
r_B^{MAX}	maximum butanol specific rate	$mg/g_{DM}h$
r_S^0	initial sugar specific consumption rate	$g/g_{DM}h$
S	substrate concentration	g/L
S_0	initial substrate concentration	g/L
X	Biomass concentration	g/L
t_A	actidogenesis phase time	h
YE	yeast extract	-
$Y_{i/S}$	sugar-to-“i-species” fractional yield coefficient	g/g
ξ_S	overall sugar conversion	-

	Buffer g/L	ξ_S	Residual Acids g/L	r_S^0 $g/g_{DM}h$	r_B^{MAX} $mg/g_{DM}h$	$Y_{X/S}$ g_{DM}/g	$Y_{B/S}$ g/g	$Y_{ABE/S}$ g/g	Max X g_{DM}/L	Max Butanol g/L	Max ABE g/L
G	0	No Growth									
	0.25	0.59	1.27	0.27	62	0.12	0.24	0.39	3.79	7.87	12.5
	0.5(*)	**0.52**	**1.13**	**0.24**	**67**	**0.11**	**0.26**	**0.42**	**3.39**	**7.90**	**12.8**
	5	0.27	4.19	0.48	49	0.14	0.08	0.11	2.03	1.12	1.5
M	0	No Growth									
	0.25	0.49	1.57	0.19	42	0.07	0.27	0.42	1.94	7.10	11.2
	0.5(*)	**0.24**	**3.99**	**0.17**	**42**	**0.15**	**0.17**	**0.23**	**2.02**	**2.18**	**2.9**
	5	0.26	5.53	0.39	20	0.15	0.09	0.12	2.12	1.25	1.7
A	0	No Growth									
	0.25	0.39	2.48	0.24	46	0.11	0.21	0.38	2.46	4.68	8.4
	0.5(*)	**0.36**	**2.90**	**0.19**	**41**	**0.13**	**0.24**	**0.42**	**2.83**	**5.15**	**8.9**
	5	0.17	5.43	0.40	31	0.17	0.05	0.07	1.62	0.43	0.7
X	0	No Growth									
	0.25	0.21	3.39	0.14	52	0.17	0.22	0.3	1.99	2.52	3.4
	0.5(*)	**0.22**	**3.34**	**0.11**	**38**	**0.17**	**0.20**	**0.28**	**2.05**	**2.48**	**3.4**
	5	0.31	5.01	0.58	89	0.06	0.15	0.21	1.07	2.51	3.5

(*) Standard medium

Table 2: Relevant data of *C. acetobutylicum* fermentation tests. $CaCO_3$: absent. Effects of buffer concentration.

	$CaCO_3$ (g/L)	ξ_S	Residual Acids g/L	r_S^0 $g/g_{DM}h$	r_B^{MAX} $mg/g_{DM}h$	$Y_{X/S}$ g_{DM}/g	$Y_{B/S}$ g/g	$Y_{ABE/S}$ g/g	Max X g_{DM}/L	Max Butanol g/L	Max ABE g/L
G	0.1	0.62	2.55	0.23	51	0.06	0.18	0.28	2.42	7.30	11.5
	0.5	0.79	3.35	0.24	77	0.03	0.15	0.24	1.61	7.28	11.5
	5	1	3.38	0.47	131	0.07	0.24	0.35	3.91	13.2	19.2
	10	1	4.91	0.43	129	0.06	0.22	0.31	3.28	12.3	17.1
M	0.1	0.55	2.04	0.14	62	0.07	0.24	0.35	2.31	7.29	10.9
	0.5	0.60	2.90	0.29	62	0.06	0.22	0.33	2.09	7.51	11.1
	5	1	5.56	0.33	98	0.05	0.16	0.22	3.03	8.91	12.4
	10	1	6.67	0.33	106	0.05	0.16	0.21	3.08	8.88	11.9
A	0.1	0.37	2.29	0.21	57	0.10	0.22	0.38	2.37	4.95	8.6
	0.5	0.92	2.43	0.25	67	0.05	0.16	0.29	2.39	8.28	15.2
	5	1	5.25	0.27	93	0.05	0.15	0.27	2.66	8.69	15.6
	10	1	5.15	0.31	118	0.05	0.17	0.30	2.77	9.62	16.8
X	0.1	0.22	2.73	0.20	68	0.16	0.26	0.36	1.92	3.10	4.4
	0.5	0.50	3.17	0.31	68	0.05	0.21	0.31	1.49	5.73	8.3
	5	0.74	4.93	0.26	43	0.10	0.19	0.26	4.28	7.82	10.8
	10	0.86	5.58	0.30	48	0.08	0.18	0.24	3.88	8.45	11.4

Table 3: Relevant data of *C. acetobutylicum* fermentation. Effects of $CaCO_3$ concentration. Data of tests carried out without $CaCO_3$ are in Table 2. Standard medium: except buffer concentration set at 0.25 g/L

	Iron g/L	$CaCO_3$ g/L	ξ_S	Residual Acids g/L	r_S^0 $g/g_{DM}h$	r_B^{MAX} $mg/g_{DM}h$	$Y_{X/S}$ g_{DM}/g	$Y_{B/S}$ g/g	$Y_{ABE/S}$ g/g	Max X g_{DM}/L	Max Butanol g/L	Max ABE g/L	B/A mol/mol
G	0.01(*)	0	0.59	1.27	0.27	62	0.12	0.24	0.39	3.79	7.87	12.5	1.63
	0.002	0	0.16	3.59	0.11	25	0.22	0.32	0.45	2.11	3.06	4.3	2.72
	0.01(**)	5	1	3.38	0.47	131	0.07	0.24	0.35	3.91	13.2	19.2	2.46
	0.002	5	0.27	4.76	0.22	47	0.14	0.28	0.37	1.56	4.50	5.9	3.20
M	0.01(*)	0	0.49	1.57	0.19	42	0.11	0.27	0.42	2.95	7.10	11.2	1.61
	0.002	0	0.38	2.39	0.05	57	0.13	0.31	0.45	2.46	5.99	8.5	2.33
	0.01(**)	5	1	5.56	0.33	98	0.05	0.16	0.22	3.03	8.91	12.4	2.59
	0.002	5	0.76	4.76	0.18	134	0.07	0.31	0.39	2.54	11.8	14.9	4.29
A	0.01(*)	0	0.39	2.48	0.24	46	0.11	0.21	0.38	2.46	4.68	8.4	1.21
	0.002	0	0.50	1.75	0.13	57	0.11	0.22	0.41	3.03	6.28	11.4	1.15
	0.01(**)	5	1	5.25	0.27	93	0.05	0.15	0.27	2.66	8.69	15.6	1.26
	0.002	5	0.93	5.15	0.13	123	0.04	0.19	0.35	2.20	9.84	18	1.17
X	0.01(*)	0	0.21	3.39	0.14	52	0.17	0.22	0.3	1.99	2.52	3.4	2.84
	0.002	0	0.19	2.92	0.25	46	0.17	0.23	0.31	1.61	2.15	2.9	2.92
	0.01(**)	5	0.74	4.93	0.26	43	0.10	0.19	0.26	4.28	7.82	10.8	2.92
	0.002	5	0.73	6.25	0.29	49	0.06	0.22	0.30	2.38	8.33	11.4	2.82

(*) standard medium

(**) Some date are reported in table 3 and duplicated here for comparisons.

Table 4: Relevant data of *C. acetobutylicum fermentation*. Effects of iron concentration. Data of tests carried out without $CaCO_3$ are in Table 2. Standard medium: except buffer concentration set at 0.25 g/L.

	$MgSO_4$ g/L	ξ_S	Residual Acids g/L	r_S^0 $g/g_{DM}h$	r_B^{MAX} $mg/g_{DM}h$	$Y_{X/S}$ g_{DM}/g	$Y_{B/S}$ g/g	$Y_{ABE/S}$ g/g	Max X g_{DM}/L	Max Butanol g/L	Max ABE g/L
G	0	0.89	5.74	0.25	47	0.05	0.22	0.34	2.79	12.6	19.1
	0.2(*)	1	3.38	0.47	131	0.07	0.24	0.35	3.91	13.2	19.2
	0.5	0.83	7.2	0.28	153	0.04	0.20	0.29	1.99	10.6	15.2
	2	0.34	5.31	0.25	105	0.12	0.13	0.18	2.42	2.73	3.8
M	0	0.91	4.48	0.25	11	0.04	0.28	0.41	2.13	13.9	20.5
	0.2(*)	1	5.56	0.33	98	0.05	0.16	0.22	3.03	8.91	12.4
	0.5	0.87	5.82	0.36	131	0.06	0.25	0.37	3.05	12.1	17.6
	2	0.81	5.91	0.12	123	0.07	0.28	0.36	3.06	11.9	15.6
A	0	0.96	4.61	0.19	126	0.04	0.22	0.44	2.25	12.7	25.5
	0.2(*)	1	5.25	0.27	93	0.05	0.15	0.27	2.66	8.69	15.6
	0.5	0.94	5.00	0.27	75	0.05	0.20	0.40	2.86	11.2	22.8
	2	0.87	5.65	0.16	77	0.06	0.22	0.39	3.41	11.4	20.5
X	0	0.79	5.06	0.13	136	0.05	0.27	0.42	1.97	11.2	17.3
	0.2(*)	0.74	4.93	0.26	43	0.10	0.19	0.26	4.28	7.82	10.8
	0.5	0.60	7.18	0.23	57	0.06	0.25	0.37	1.93	7.91	12.1
	2	0.69	6.11	0.20	65	0.08	0.21	0.29	2.74	7.60	10.4

(*) standard concentration

Table 5: Relevant data of *C. acetobutylicum* fermentation. Effects of $MgSO_4$. Standard medium: except buffer concentration set at 0.25 g/L. CaCO3 concentration: 5 g/L.

	$MnSO_4$ g/L	ξ_S -	Residual Acids g/L	r_S^0 $g/g_{DM}h$	r_B^{MAX} $mg/g_{DM}h$	$Y_{X/S}$ g_{DM}/g	$Y_{B/S}$ g/g	$Y_{ABE/S}$ g/g	Max X g_{DM}/L	Max Butanol g/L	Max ABE g/L
G	0	0.75	6.83	0.24	122	0.05	0.21	0.30	2.22	9.98	14.2
	0.01	1	3.38	0.47	131	0.07	0.24	0.35	3.91	13.2	19.2
	0.1	0.76	7.20	0.27	67	0.06	0.22	0.31	2.95	10.3	14.2
M	0	0.76	6.44	0.15	83	0.08	0.21	0.29	3.27	8.51	11.7
	0.01	1	5.56	0.33	98	0.05	0.16	0.22	3.03	8.91	12.4
	0.1	0.96	5.01	0.16	134	0.07	0.29	0.44	3.72	14.6	21.8
A	0	0.84	5.60	0.20	88	0.07	0.16	0.28	3.26	7.71	13.7
	0.01	1	5.25	0.27	93	0.05	0.15	0.27	2.66	8.69	15.6
	0.1	0.91	5.75	0.13	100	0.07	0.21	0.38	3.60	11.2	20
X	0	0.76	6.51	0.12	97	0.06	0.17	0.24	2.39	7.10	9.7
	0.01	0.74	4.93	0.26	43	0.10	0.19	0.26	4.28	7.82	10.8
	0.1	0.93	4.76	0.20	130	0.10	0.28	0.41	4.64	13.7	20

Table 6: Effects of $MnSO_4$ on the growth, the production of solvents and acids, the consumption of sugar and the fermentation yields.

4.2 Biobutanol Production from Hexose and Pentose Sugars

Detailed results are reported elsewhere (Raganati et al., Butanol production from hexose and pentose sugars, 2014, submitted for publication). Main results of the study are reported in this section.

The *C. acetobutylicum* fermentation adopting binary solutions of sugars typically present in the lignocellulosic biomass hydrolysates has been successfully investigated. The fermentation tests regarded the binary mixtures of sugars made of: glucose/mannose (GM), glucose/arabinose (GA), glucose/xylose (GX), mannose/arabinose (MA), and arabinose/xylose (AX). Tests have pointed out that *C. acetobutylicum* is able to simultaneously metabolize the investigated sugars (glucose, mannose, arabinose, and xylose) and no diauxic growth has been observed.

Except for glucose, the maximum concentration of butanol measured during tests with the binary mixtures was higher than that assessed during the test with single sugars. It was close to that assessed for glucose fermentation.

4.3 Butanol Production From High Sugar Content Beverages

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ABSTRACT

The aim of this contribution is twofold: i) to propose a potential disposal process for high sugar content beverage (HSCB) industries; ii) to contribute to the study of butanol production by *Clostridium acetobutylicum* adopting no-editable feedstocks. HSCBs were adopted as renewable feedstock for butanol production by *C. acetobutylicum* DSM 792. Four types of commercial beverages were investigated: fruit juices (pineapple and pear), syrups (lemon and almond), two soft drinks, and one sport drink. The beverages contain high levels of sugars – between 50 and 1000 g/L - and their disposal is a critical issue for beverage industries.

The HSCB conversion process under batch conditions was characterized in terms of metabolites and cell production, and sugar conversion. Initial overall sugar concentration was set no larger than about 120 g/L. *C. acetobutylicum* was able to grow on HSCBs supplemented with other nutrients. The sucrose hydrolyses improved the fermentation performances in terms of solvent production and sugar conversion (up to about 100%). Under optimized conditions butanol concentration and butanol yield were: pineapple-juice) 13.3g/L, 0.16g/g; pear-juice) 12.8g/L, 0.14g/g; lemon syrup) 13.2g/L, 0.19g/g; almond-syrup) 13.8g/L, 0.14g/g; commercial soft drinks) 13-14g/L, 0.17-0.18g/g; sport dink) 5.9g/L, 0.11g/g. The maximum butanol production rate was 0.12g/Lh.

Keywords: butanol, *Clostridium acetobutylicum*, beverages disposal, fructose, sucrose.

INTRODUCTION

Over the last decade the depletion of oil resources and the concern regarding both economic and environmental issues associated with petroleum-based fuels have renewed the interest for biofuels produced from renewable resources (Antoni et al., 2007; Friedl, 2012). The production processes of biofuels from waste(water)s are still more scheming because they fulfil a remediation request too (Dürre, 2007): a critical issue for industries from both technical and cost point of view.

Industrial and academic researches have paid attention to the development of (bio)sustainable processes and to the adoption of renewable feedstocks. The spectrum of biofuels includes the butanol, a simple four carbon alcohol characterized by interesting features (Dürre, 2007;Cascone, 2008; Lee et al., 2008a): low vapour pressure, blending with both gasoline and diesel at any fraction, energy content close to that of the gasoline, possibility to fuel current configurations of engines without any retrofitting (Dürre, 2007). Butanol may be produced from renewable resources (biomass) by the acetone butanol ethanol (ABE) fermentation process (Jones and Woods, 1986).

ABE process typically occurs during the later stage of batch fermentations of some *Clostridium* strains - saccharolytic butyric acid-producing bacteria - under selected operating conditions (*Clostridium saccharoperbutylacetonicum, Clostridium acetobutylicum, Clostridium beijerinckii, Clostridium aurantibutyricum*). These strains are able to metabolize a great variety of substrates: pentoses, hexoses, mono-, di- and poly-saccharides (Flickinger and Drew, 1999). Batch fermentation of solvent-producing *Clostridium* strains proceeds following a two stage process: acidogenesis and solventogenesis phases (Jones and Woods, 1986). The acidogenesis phase is characterized by production of cells, hydrogen, carbon dioxide, acetic acid, and butyric acid. As the acid concentration increases (pH decrease), the cell metabolism shifts to solvent production (solventogenesis) and acidogenic cells – able to reproduce themselves - shift to the solventogenesis state with a morphological change. The solventogenesis phase is characterized by endospores unable to reproduce themselves and by the production of solvents. Two different physiological states must be then taken into account for *Clostridia*: one for the acidogenic phase, and one for the solventogenic phase.

Major challenges for the ABE industrial fermentation regard four issues: i) the butanol toxicity to microbial cells; ii) the butanol productivity; iii) the cost of fermentation feedstocks; iv) the cost of the butanol recovery from the fermentation broth (Napoli et al., 2012a; Napoli et al., 2012b). Recent developments of molecular techniques applied to solventogenic microorganisms in combination with advances in fermentation technology and downstream processing have contributed to improve feasibility and competitiveness of the ABE fermentation process. The ABE concentration and productivity – assessed as butanol concentration divided by the time of the fermentation - in batch fermentations by solventogenic *Clostridium* species can be increased up to 13-18 g/L and 0.2-0.3 g/(L·h), respectively (Ezeji and Blaschek, 2008). Modest increase in ABE titer and productivity can be achieved by supplementing acetate (Chen and Blaschek, 1999) or butanol (Junne, 2010; Junne et al., 2006) to the fermentation medium.

Fermentation substrate is a key factor of the cost of butanol production (Qureshi and Blaschek, 2000; Kumar and Gayen, 2011; Toerien and Hattingh, 1969). The feedstock cost drastically reduces when waste materials may be adopted. Several typologies of waste(water)s have been tested as feedstocks (Ezeji and Blaschek, 2008; Ezejiet al., 2007c; Liu et al., 2010; Napoli et al., 2010; Dwidar et al., 2012; Raganati et al., 2013). Results have been very promising and promoted the investigation of a wider the spectrum of this typology of feedstocks.

High sugar content beverages (HSCBs) - such as fruit juices, syrups, soft drinks, and sport drinks - may be a potential carbon source for the ABE fermentation. They contain sugars as sucrose, fructose, and glucose. Even though the fate of these beverages is to refresh people, a huge quantity of them are yearly disposed. As a matter of fact, the disposal of industrial wastewater streams, seizures of illegal goods, and expiry date beverages is a critical issue for the high carbon content. Dwidar et al. (2012) adopted carbonated beverages as carbon source to successfully produce bioethanol. The preliminary tests to produce butanol from a carbonated beverage were very promising. To author knowledge, a thorough characterization of butanol production by adopting high sugar content beverages is still absent in the literature.

The present study reports results of the ABE fermentation by *Clostridium acetobutylicum* DSM 792 using HSCBs as substrate. Four types of commercial beverages were tested: fruit juices (pineapple and pear juices), syrups (lemon and almond syrups), two commercial soft drinks, and one sport drink. The overall sugar concentration of the beverages ranged between 50 and 1000 g/L. Initial overall sugar concentration was set no larger than about 120 g/L. The fermentations were characterized in terms of overall sugar conversion, maximum butanol concentration, butanol yield, and butanol production rate.

MATERIALS AND METHODS

Microorganism

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Stock cultures were reactivated according to the DSMZ procedure. Reactivated cultures were stored at -80°C. The thawed cells were inoculated into 15 mL Hungate tubes containing 12 mL synthetic medium: 30 g/L glucose, 5 g/L yeast extract (YE) and P2 stock solution (Qureshi and Blaschek, 1999). Cells were grown under anaerobic conditions for 48 h at 37°C. Then pre-cultures were transferred into fermentation bottles.

Medium

Synthetic medium: glucose, fructose, and sucrose as carbon source

The synthetic medium consisted of 5 g/L YE, 2 g/L ammonium chloride (Nitrogen Source) and 5 g/L $CaCO_3$ supplemented with P2 stock solution: buffer) 0.25 g/L KH_2PO_4, 0.25 g/L K_2HPO_4; mineral) 0.2 g/L $MgSO_4 \cdot 7H_2O$, 0.01 g/L $MnSO_4 \cdot 7H_2O$, 0.01 g/L $FeSO_4 \cdot 7H_2O$ (Qureshi and Blaschek, 1999). It was sterilized in autoclave prior to the carbon source addition. Chemicals ($CaCO_3$, KH_2PO_4, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$) and YE were supplied by Sigma Aldrich.

Glucose, fructose and sucrose (Sigma Aldrich) were investigated because they are typically present in HSCBs. Concentrated solutions of sugars were sterilized by filtration and supplemented to the autoclaved synthetic medium. The stock solution concentration of each investigated sugar was 300 g/L. The initial concentration of each sugar in the fermentation tests ranged between 5 and 100 g/L.

High sugar content beverages

Table 1 lists the tested HSCBs characterized in terms of pH and concentration of sugars. They were selected among the most popular beverages: two fruit juices, two concentrated syrups (adopted to prepare drinks), two soft-drinks and a sport drink. The pear juice and the syrups were diluted – 2:3 and 1:10, respectively – before to be fermented. All the beverages were neutralized at pH=7 and then sterilized in autoclave.

Fermentation tests were carried out with a HSCB supplemented with nutrients (HSCB+). The medium consisted of a HSCB, 5 g/L YE, 2 g/L ammonium chloride, 5 g/L $CaCO_3$, and P2 stock solution. The pH was adjusted to 7. The medium was sterilized in autoclave.

Hydrolyzed HSCBs were also adopted as carbon source for some fermentation tests (HHSCB+). Hydrochloric acid was supplemented to the HSCB until pH=1.5 established, then the solution was autoclaved (120°C for 20 minutes). Except for the carbon source, the composition of the medium was that adopted for the HSCB+. The sport drink was not hydrolysed because the sucrose concentration was quite low.

Analytical Procedures

The pH was measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments). Analysis of culture samples withdrawn from fermenters provided concentration in the liquid phase of biomass, sugars and metabolites, total organic carbon (TOC), and total nitrogen (TN). 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 OD_{600} = 0.4 g_{DM}/L (Napoli et al., 2011). The cell concentration was not assessed in culture samples from the fermentation of the almond syrup because the turbidity of the beverage interfered the measurement.

The concentration of soluble species was measured in the liquid phase after cell harvesting by centrifugation (10,000 rpm for 10 min). Sugar concentration was measured by HPLC (Agilent 1100 system). The sugars were separated on a 8 µm Hi-Plex H (30 cm × 7.7 mm) column at room temperature and detected with a refractive index detector. Deionized water was used as mobile phase at 0.6 mL/min flow rate. An Agilent 6890 GC apparatus equipped with a Poraplot Q capillary column (25 m × 0.32 mm) and a FID was used for the metabolite analysis. Hexanoic acid was used as internal standard to analyse acids and alcohols and to assess their concentrations. A Shimadzu TOC 5000A analyzer was used to measure TOC/TN concentration.

Apparatus and Operating Conditions

Pyrex screw capped bottles (100 mL) containing 75 mL medium were used as fermenters. The medium was inoculated with 6.25% (v/v) suspension of active growing pre-cultures. 3 mL of cultures were sampled periodically for cell/metabolites characterization. Each test was carried out in triplicate.

Tests were carried out in fermenters at rest, at 37 °C, without pH control.

Batch fermentations were characterized in terms of cell growth, pH, sugar conversion, and acids and solvents production. Measurements were worked out to assess the following data:

- overall sugar conversion (ξ_S) - the ratio between the concentration of converted sugar and the initial sugar concentration;

- sugar-to-“i-species” fractional yield coefficient ($Y_{i/S}$), - the ratio between the incremental “i-species” (cell or butanol) mass and the decrease of the substrate mass measured over the same time-interval;
- the initial sugar specific consumption rate (r_S^0) – it was estimated at the beginning of the exponential phase as the slope of the sugar concentration vs. time plot (see Fig. 1) , divided for the cell concentration measured at the exponential phase onset;
- the maximum butanol specific rate (r_B^{MAX}) - it was estimated at the threshold of the solventogenesis phase as the slope of the butanol concentration vs. time plot (see Fig. 1), divided for the cell concentration measured at the solventogenesis onset;
- the maximum concentration of butanol, ABE, and cells measured during the batch fermentation;
- the butanol production rate (R_B) – it was the 95% of the maximum butanol concentration divided the fermentation time until the 95% of the maximum butanol concentration;
- the residual acid concentration measured at the end of the solventogenesis phase.

RESULTS AND DISCUSSION

Glucose, fructose and sucrose. Batch fermentation tests were carried out adopting synthetic medium supplemented with a sugar - glucose, fructose and sucrose - as carbon source.

Fig. 1 reports the time resolved profiles of the concentration of *C. acetobutylicum* cells, sucrose (initial concentration 60 g/L), and metabolites (acetic acid, butyric acid, acetone, butanol and ethanol) as well as of pH, measured during a batch culture.

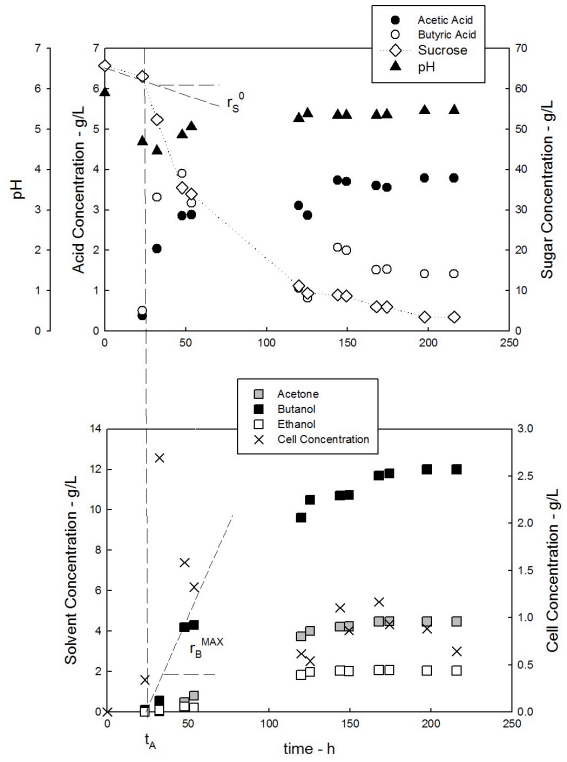

Fig. 1: Data measured during *C. acetobutylicum* fermentation in standard medium supplemented with sucrose (nominal initial concentration: 60 g/L). The vertical dashed line marks the beginning of the solventogenesis phase.

The analysis of the data confirmed the typical two-phase behaviour of the fermentation (Jones and Woods, 1986): acidogenesis phase and solventogenesis phase. The acidogenesis phase was characterized by: i) continuous conversion of the substrate; ii) steady increase of concentration of cells and acids; iii) pH decrease. As the pH approached 4 (t_A=25 h), solventogenesis phase established (Napoli et al., 2011). The solventogenesis phase was characterized by: i) gradual decrease of the sugar concentration approaching a stationary value; ii) gradual decrease of the cell concentration as a consequence of cell lysis (Ezeji et al., 2007a; Mutschlechner et al., 2000; Napoli et al., 2009); iii) steady increase of concentration of solvents approaching a stationary value; iv) gradual decrease of the acids concentration as a consequence of their conversion by *Clostridium* cells at a rate larger than their production.

The initial sugar specific consumption rate (r_S^0) and the maximum butanol specific rate (r_B^{MAX}) were 0.34 g/(g_{DM}h) and 0.087 g/(g_{DM}h), respectively (Table 2). It is worth to note that both data underestimate the true values because the periodic sampling of the cultures did not allow an instantaneous assessment of the rates. An accurate assessment of the rate would be possible by adopting continuous cultures (Napoli et al., 2012b; Napoli et al., 2011; Olivieri et al., 2010).

Figure 2 reports the sugar concentration and the ABE (acetone + butanol + ethanol) concentration measured during the batch tests carried out with the fermentation medium supplemented with the investigated sugars: Fig. 2A and Fig 2B refer to tests carried out with a single sugar; Fig. 2C e Fig 2D refer to the test carried out with a mixture of the three investigated sugars. Table 2 reports relevant data regarding the fermentation tests.

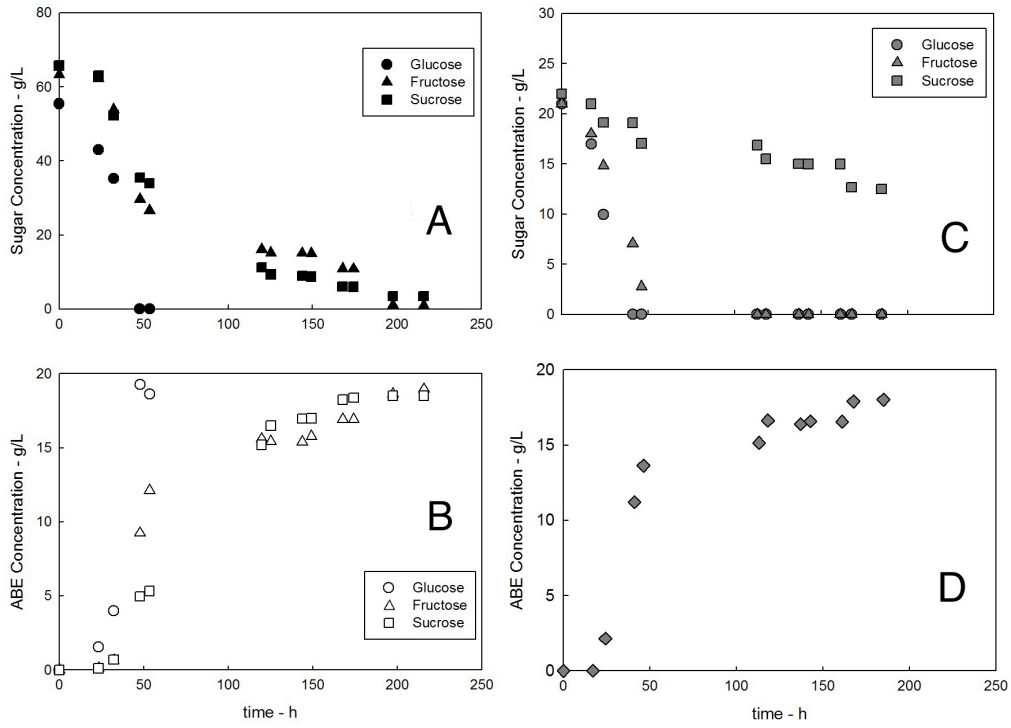

Fig. 2: Residual sugar concentration and ABE concentration measured during batch fermentation. A and B refer to tests with a single sugar (glucose, fructose, and sucrose). C and D refer to tests with a sugar mixture.

Results of the analysis of Figures 2A and 2B and of data reported in Table 2 are reported and discussed hereinafter.

C. acetobutylicum was able to grow on the investigated sugars. The glucose was completely converted (x_S=1), fructose and sucrose were present at the end of the fermentation (x_S =0.98 and 0.95, respectively). ABE was produced. The fermentation performances expressed in terms of production rate and production yield of solvents depended on the sugar. Tests carried out with glucose confirmed the high performance typically reported for this sugar: 19.2 g/L of ABE produced within 50 h, $Y_{X/S}$=0.07, $Y_{B/S}$=0.24, $Y_{ABE/S}$=0.35. The acidogenesis phase lasted less than 1 day. The acetone:butanol:ethanol molar ratio was 3:6:1. The residual acid concentration at the end of fermentation was 3.38 g/L. The fermentation performances assessed for tests carried out adopting fructose were similar to those assessed for tests carried out adopting glucose. The sucrose fermentation, on the contrary, was characterized by lower performances with respect to those assessed for glucose fermentation.

The ABE production depends on the sugar. In particular, the following features depend on the sugar: i) the acidogenesis phase time (t_A); ii) the solvent production time (assumed as the time interval during which solvents were accumulated in the broth); iii) the final concentration of ABE. The first two features decreased with the sugar species with the order sucrose, fructose, and glucose. The last feature decreased with the sugar species with the order glucose, fructose, and sucrose.

The residual acid concentration and the butanol yield depended on the sugars (Table 2). It is interesting to note that tests carried out with sucrose were characterized by both high residual acid concentration and low ABE yield. It appears that the capacity of *C. acetobutylicum* to convert acids into ABE is lower when sucrose is adopted as carbon source than that measured when glucose/fructose are adopted.

The effect of sugar concentration was investigated for all the tested sugars. The initial sugar concentration ranged between 5 and 100 g/L. The analysis of results (data not reported) pointed out that the amount of converted sugar during the acidogenesis phase depended on the sugar. The drop of sugar concentration during the acidogenesis phase was about 6, 10 and 15 g/L for glucose, fructose, and sucrose, respectively. Obviously, tests carried out at initial sugar concentration lower that these figures were characterized by absence of solventogenesis phase (Napoli et al., 2009). Therefore, the amount of the carbon source required for solvent production depends on the sugar and it increases with the order glucose-fructose-sucrose.

Fig. 3 reports r_B^{MAX} as a function of the sugar concentration (C_S) at the solventogenesis onset. The analysis of Fig. 3 suggests that r_B^{MAX} may be described by a Monod-like model:

$$r_B^{MAX} = B^{MAX} \cdot \frac{C_S}{k_d + C_S} \quad (1)$$

where k_d is the sugar concentration at which r_B^{MAX} is half of its maximum value B^{MAX}. Table 3 reports k_d and B^{MAX} for each sugar assessed by working out data in Fig. 3.

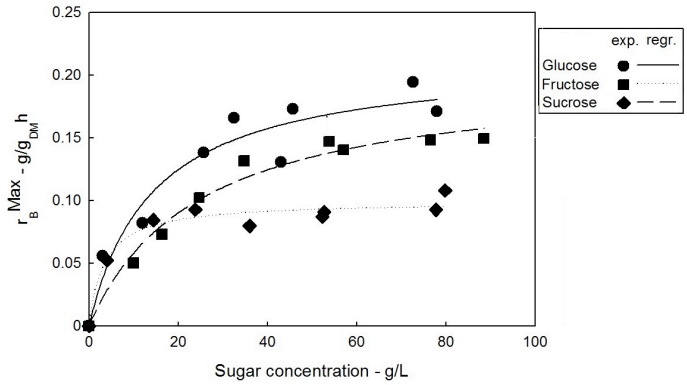

Fig. 3: Maximum specific production rate of butanol at the onset of the solventogenesis phase in batch cultures as a function of sugar concentration.

B^{MAX} decreased with the sugar species with the order glucose-fructose-sucrose. k_d was of the order of 10 g/L whatever the sugar. It is interesting to note the assessed values of k_d and B^{MAX} are in agreement with those found for lactose (Napoli et al., 2009): k_d= 28 g/L and B^{MAX}=0.13 $g_B/(g_{DM}h)$.

Table 3: B^{MAX} and k_d assessed according to Eq. (1) for each sugar.

	Glucose	Fructose	Sucrose
B^{MAX}, $g_B/(g_{DM}h)$	0.23	0.20	0.099
k_d, g/L	18	24	3

The analysis of the results of fermentation tests carried out with a mixture of sugars is now in order. Tests were carried out with a mix of glucose, fructose, and sucrose (GFS) at mass ratio of 1:1:1 (total initial sugar concentration=60 g/L). Results reported in Table 2 and Fig.s 2C and 2D point out that:

- the final concentration of ABE was 18 g/L ABE;
- the sugars (glucose, fructose and sucrose) were simultaneously converted during the fermentation, although the rate of sugar utilization was sugar specific (Fig. 2C). The initial glucose uptake rate (0.24 $g/g_{DM}h$) was about that assessed during tests carried out with a 20 g/L glucose medium. The initial fructose conversion rate decreased of about 17 % with respect to that assessed during the reference test. The initial sucrose uptake rate decreased of about 83 % with respect to that assessed during the reference test. The observed behaviour is a result of the combined effects of the sugars that dump both fructose and sucrose uptake.
- fructose and glucose were completely converted and only 43% of sucrose was metabolized (sucrose residual concentration 12 g/L).

The maximum specific butanol production rate was about 0.090 $g/(g_{DM}h)$. The rate was about that assessed by means of Eq. (1) (0.10 $g/(g_{DM}h)$) for glucose at the concentration measured at the solventogenesis onset (15 g/L). The butanol production rate for fructose and sucrose at the solventogenesis onset concentration – 14 and 20 g/L, respectively – is 0.074 and 0.086 $g/(g_{DM}h)$. As expected, the butanol production rate at the solventogenesis onset was controlled by the most fermentable sugar.

Altogether, the performances of GFS fermentation were worse when compared to the glucose and fructose fermentation at the same overall initial sugar concentration (60 g/L).

As a result of the investigation it was highlighted that *C. acetobutylicum* was able to metabolize all the sugars typically present in high sugar content beverages.

High Sugar Content Beverages. Table 4 reports the results of the batch fermentation tests carried out using a HSCB as carbon source. Tests carried out inoculating *C. acetobutylicum* into the raw HSBCs pointed out that the microorganism didn't grow because the lack of some indispensable nutrients in the fermentation broth. HSCB+ refer to tests carried out inoculating *C. acetobutylicum* into a high sugar content beverage supplemented as described in the Material and Method section.

Fig. 4 reports the time resolved profiles of the concentration of *C. acetobutylicum* cells, sugars, (glucose, fructose, and sucrose), and metabolites (acetic acid, butyric acid, acetone, butanol, and ethanol) as well as of pH, measured during a batch culture adopting a sport drink as carbon source. The concentration of sugars at the beginning of the test was enough to drive the fermentation into the solventogenesis phase. As expected, the conversion of glucose and fructose was practically total and the sucrose conversion was quite low. The maximum butanol production rate was 0.11 g/(g_{DM}h) (Table 4) and it was about that assessed by means of Eq. (1) (0.13 g/(g_{DM}h)) for glucose at the concentration measured at the solventogenesis onset (28 g/L). The butanol production rate for fructose and sucrose at the solventogenesis onset concentration – 28 and 45 g/L, respectively – is 0.11 and 0.092 g/(g_{DM}h). The comparison of the maximum butanol production rates confirms that at the solventogenesis onset the butanol production was controlled by the most fermentable sugar.

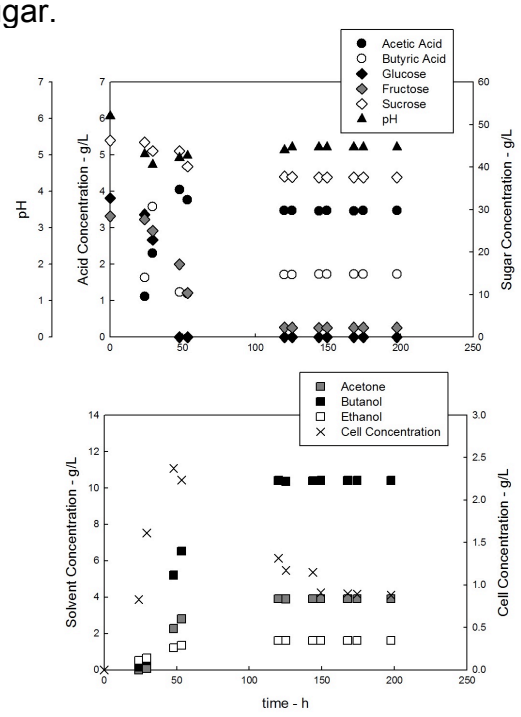

Fig. 4: Data measured during *C acetobutylicum* fermentation in supplemented sport drink (HSCB+).

Fig. 5 A-B report the concentration of ABE measured at the end of the fermentation and sugar concentration measured at the beginning (t^0) and at the end of the fermentation (t^F) for tests carried out with supplemented HSCB.

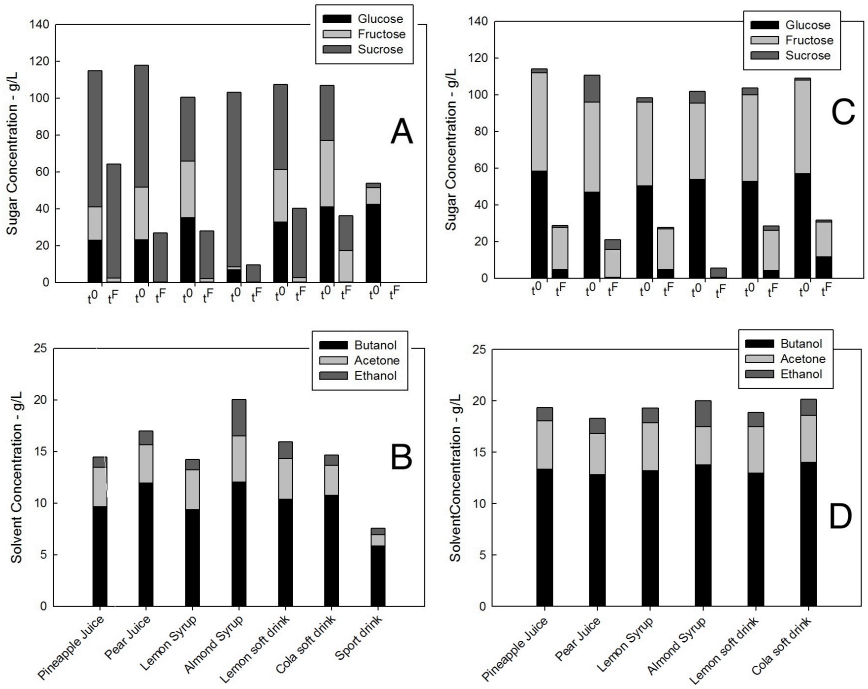

Fig. 5: ABE concentration measured at the end of the fermentation and sugar concentration measured at the beginning (t^0) and at the end of the fermentation (t^F). A and B refer to tests with a HSCB+. C and D refer to tests with HHSCB+.

The analysis of Table 4 and Fig. 5.B suggests that *C. acetobutylicum* efficiently grows on all the investigated HSCBs. Except for the sport drink fermentation, the final concentration of butanol was about 10 g/L. The sport drink fermentation was characterized by depletion of all sugars and the butanol concentration stopped just at 6 g/L. The butanol and the ABE yields were quite interesting and in particular those assessed for tests carried out with the pineapple juice (0.19 and 0.28 g/g respectively). Except for almond syrup fermentation, the r_B^{MAX} was about 0.10 g/(g_{DM}h) whatever the HSCB+ investigated. The r_B^{MAX} was not reported for almond syrup because the turbidity of the medium was too high to measure the cell concentration.

The glucose and the fructose were almost completely converted (more than 90 %) during the fermentation, whatever the HSCB+. A significant amount of sucrose was found unconverted at the end of the fermentation: the maximum sucrose conversion degree was 25% for lemon syrup. Even though the observation was in agreement with the results reported for reference sugars, the amount of unconverted sugar could reduce the advantage of the ABE fermentation. Therefore, tests were carried out with hydrolysed HSCB+ (HHSCB+) to pre-convert the sucrose in more fermentable sugars. The Table 1 reports the sugar composition of the investigated HSCB after hydrolysis: almost all the sucrose is completely hydrolysed in fructose and glucose. The analysis of Figures 5C and 5D and Table 4 points out that the performance of the fermentation tests carried out with hydrolysed HSBC are better than that found for HSBC+ tests. A significant increase of the final butanol concentration - larger than 13 g/L - was measured. The total sugar conversion degree was larger than 70 %, whatever the beverage. The butanol production rate was very interesting – order of 0.1 g/Lh - being comparable with those obtained by high performance strain/substrate systems, 0.2-0.3 g/(L·h) [10].

CONCLUSIONS

Batch fermentations of *Clostridium acetobutylicum* adopting high sugar content beverages (HSCBs: fruit juices, fruit syrups, soft drinks, sport drink) were successful. Main results were:

- *C. acetobutylicum* did not grow on raw HSCBs;
- *C. acetobutylicum* grew on HSCBs supplemented with yeast extract and mineral salts. The butanol maximum concentration was ≈10g/L. Glucose and fructose conversion was practically complete. Sucrose conversion was very low;
- tests carried out with supplemented hydrolysed HSCBs were characterized by solvents production and sugar conversion degree higher than those measured during just supplemented HSCBs.

Results pointed out that HSCBs are a promising substrate for the ABE fermentation process and that they may be converted almost completely. Considering the huge amount of HSCBs that should be disposed in each country, the investigated process has a twofold advantage: to reduce the disposal cost for these kind of beverages and to produce butanol that may be adopted as biofuel.

NOMENCLATURE

ABE	acetone-butanol-ethanol
B	butanol
DM	dried mass
HSCB	high sugar content beverage
HSCB+	supplemented high sugar content beverage
HHSCB+	supplemented hydrolysed high sugar content beverage
S	sugar
X	biomass
YE	yeast extract
B^{MAX}	maximum r_B^{MAX}-value, mg/(g_{DM}h)
C	concentration, g/L
k_d	parameter in Eq. (1), g/L
r_B^{MAX}	maximum butanol specific rate, mg/(g_{DM}h)
R_B	butanol production rate (g/Lh)
r_S^0	initial sugar specific consumption rate, g/(g_{DM}h)
t^0	initial time, h
t^F	final time, h
t_A	time of the acidogenesis phase, h
$Y_{i/S}$	sugar-to-“i-species” fractional yield coefficient, –
ξ_S	overall sugar conversion degree, –

Table 1: High sugar content beverages investigated: pH and sugars concentration.

HSCB		Initial pH	Glucose-fructose-sucrose initial concentration (g/L)
Pineapple juice	HSCB	2.5-3	23-18-74
	HHSCB+		58-54-2
Pear juice	HSCB	2.5-3*	23-28-66**
	HHSCB+		47-49-15**
Lemon syrup	HSCB	2.5-3*	35-31-35**
	HHSCB+		50-46-2**
Almond syrup	HSCB	5.5-6*	7-2-95**
	HHSCB+		54-42-6**
Lemon soft-drink	HSCB	2.5-3	33-28-46
	HHSCB+		53-47-3
Cola soft-drink	HSCB	2.5-3	41-36-30
	HHSCB+		57-51-1
Sport drink	HSCB	2.5-3	42-9-2

**before dilution*

***after dilution (1:10 for the syrups and 2:3 for the pear juice)*

Table 2: Relevant data of *Clostridium acetobutylicum* fermentation tests carried out adopting glucose (G), fructose (F) and sucrose (S) as carbon source. Nominal initial concentration of sugar(s): 60 g/L.

	ξ_S, –	Residual acids, g/L	r_S^0, g/(g_{DM}h)	r_B^{MAX}, g/(g_{DM}h)	R_B g/(Lh)	$Y_{X/S}$, g_{DM}/g	$Y_{B/S}$, g/g	$Y_{ABE/S}$, g/g	Max C_X, g_{DM}/L	Max C_B, g/L	Max C_{ABE}, g/L
G	1	3.4	0.47	0.13	0.278	0.07	0.24	0.35	3.91	13.2	19.2
F	0.98	4.2	0.40	0.15	0.073	0.04	0.21	0.31	2.71	12.8	18.9
S	0.95	5.2	0.34	0.087	0.069	0.04	0.19	0.30	2.69	12.0	18.5
GFS	0.84	5.3	0.48	0.090	0.072	0.05	0.24	0.34	2.80	12.4	18.0

Table 4: Relevant data of *C. acetobutylicum* fermentation tests using HSCB as carbon source.

		ξ_S, –	Residual acids, g/L	r_S^0, g/(g_{DM}h)	r_B^{MAX}, g/(g_{DM}h)	R_B g/(Lh)	$Y_{X/S}$, g_{DM}/g	$Y_{B/S}$, g/g	$Y_{ABE/S}$, g/g	Max C_X, g_{DM}/L	Max C_B, g/L	Max C_{ABE}, g/L
Pineapple juice	HSCB	NO GROWTH										
	HSCB+	0.44	3.73	0.44	0.11	0.071	0.04	0.19	0.28	1.98	9.65	14.6
	HHSCB+	0.75	4.00	0.15	0.10	0.097	0.03	0.16	0.23	2.98	13.3	19.4
Pear Juice	HSCB	NO GROWTH										
	HSCB+	0.77	5.28	0.27	0.080	0.067	0.06	0.13	0.18	5.45	11.9	16.9
	HHSCB+	0.82	5.52	0.20	0.084	0.073	0.07	0.14	0.20	6.05	12.8	18.3
Lemon syrup	HSCB	NO GROWTH										
	HSCB+	0.72	3.73	0.46	0.12	0.071	0.03	0.13	0.20	1.96	9.40	14.2
	HHSCB+	0.72	3.93	0.22	0.10	0.096	0.04	0.19	0.27	2.97	13.2	19.3
Almond syrup	HSCB	NO GROWTH										
	HSCB+	0.91	2.54	\	\	0.076	\	0.13	0.21	\	12.0	20.0
	HHSCB+	0.95	2.56	\	\	0.103	\	0.14	0.21	\	13.8	20.0
Lemon soft-drink	HSCB	NO GROWTH										
	HSCB+	0.65	5.20	0.28	0.11	0.086	0.03	0.15	0.23	2.37	10.4	15.9
	HHSCB+	0.73	6.54	0.19	0.11	0.088	0.03	0.17	0.25	2.07	13.0	18.9
Cola soft-drink	HSCB	NO GROWTH										
	HSCB+	0.66	5.6	0.25	0.10	0.062	0.05	0.15	0.21	2.96	10.7	14.7
	HHSCB+	0.72	5.2	0.24	0.11	0.080	0.05	0.18	0.26	4.01	14	20.1
Sport-drink	HSCB	NO GROWTH										
	HSCB+	1	5.6	0.48	0.086	0.119	0.06	0.11	0.14	3.02	5.87	7.57

5 KINETIC MODELS

This chapter reports about a kinetic dynamic model and MFA adopted to characterize the ABE fermentation. Batch fermentation tests were carried out with an initial sugar concentration of 60 g/L, then the experimental data were used to perform MFA and to implement the kinetic model.

A kinetic dynamic model of acetone–butanol–ethanol (ABE) production by *Clostridium acetobutylicum* DSM 792 has been developed by adopting the biochemical networks simulator COPASI. The effect of sugars has been studied implementing the model with different sugars: glucose, mannose, fructose, sucrose, lactose, xylose and arabinose.

The MFA has been adopted to investigate the role of the main reaction steps of the *C. acetobutylicum* metabolic pathway to convert reference sugars (glucose and xylose) of hydrolyzed lignocellulosic biomass into butanol.

5.1 Analysis of Substrate Effects on Butanol Production by *Clostridium acetobutylicum* by means of a Kinetic Dynamic Model

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A kinetic dynamic model of acetone–butanol–ethanol (ABE) production by *Clostridium acetobutylicum* DSM 792 was proposed using the biochemical networks simulator COPASI. The effects of substrate were studied applying the model at several sugars: glucose, mannose, fructose, sucrose, lactose, xylose, and arabinose. The metabolic pathway was characteristic for the investigated sugar. The Embden-Meyerhof-Parnas (EMP) pathway equations were adopted for hexoses and disaccharides, the pentose phosphate (PP) pathway equations were adopted for pentoses.

Batch fermentation tests were carried out at 60 g/L initial sugar concentration. The experimental data were elaborated to assess the kinetic parameters of the model.

The proposed model gave satisfactory results for each tested sugar: the squared correlation coefficient (r^2) of metabolite concentrations assessed during tests and simulated ones ranged between 0.87 and 0.925.

Keywords: butanol, *Clostridium acetobutylicum*, dynamic model, kinetic parameters, COPASI

INTRODUCTION

The use of lignocellulosic biomass as a renewable energy source is at the center of social/industrial attention because it may be a satisfactory answer to the world energy request and to the control of the environmental impact related to the conversion of energy vectors. Lignocellulosic biomasses fixs carbon dioxide and

sunlight at quite high rate and may be adopted as renewable feedstoks to produce energy vectors according to several routes (Dürre, 2007). They may be adopted to produce (bio)gas, (bio)oil, ethanol, butanol, etc.. However, no-one of these routes is aready at industrial scale.

Butanol is an energy vector characterized by remarkable features - hydrophobicity, high energy density, possibility to be adopted in the current four-stroke engines without any upgrade, distribution by the current infrastructurs – and has been proposed as a substitute/supplement of gasoline as transportation fuel (Cascone, 2008; Dürre, 2007; Lee et al., 2008). Acetone–butanol–ethanol (ABE)-producing clostridia could produce solvents by fermentation of several biomasses, such as palm oil waste (Lee et al., 1995), agro-industrial waste (Raganati et al., 2013), and agricultural crops (Qureshi et al., 2001). Considerable research has been carried out on the ABE fermentation system for high butanol production (Tashiro et al., 2004; Napoli et al., 2010; Napoli et al., 2012). *Clostridium saccharoperbutylacetonicum*, *C. acetobutylicum*, *C. beijerinckii*, and *C. aurantibutyricum* are able to metabolize a great variety of substrates, pentoses, hexoses, mono-, di- and polysaccharides (Jones and Woods, 1986). Under batch conditions the fermentation process of solvent-producing Clostridium strains proceeds with the production of cells, hydrogen, carbon dioxide, acetic acid and butyric acid during the initial growth phase (acidogenesis) (Jones and Woods, 1986). As the acid concentrations increases (pH decreases), the metabolism of cells shifts to solvent production (solventogenesis) and acidogenic cells – able to reproduce themselves - shift to the solventogenesis state with a morphological change (Jones and Woods, 1986). During solventogenesis the active cells become endospores unable to reproduce themselves. According to the fermentation path, two physiological states must be taken into account for *Clostridia*: one for the acidogenic phase, and one for the solventogenic phase.

The complexity of the metabolic pathway involved in ABE production is testified by the very few models reported in the literature; Papoutsakis (1984) developed a stoichiometric model: it may be used to calculate or estimate the rates of reactions occurring within the pathway in several ABE-producing clostridia. Votruba et al. (1986) developed a mathematical model for batch cultures of *Clostridium acetobutylicum* based on biochemical as well as physiological aspects of growth and metabolite synthesis. Desai et al. (1999) analysed the contribution of acid formation pathways in the metabolism of *C. acetobutylicum* ATCC824^T according to the metabolic flux analysis (MFA). Shinto et al. (2007, 2008) reported a kinetic simulation model to describe the dynamic behaviour of metabolites in ABE production by *C. saccharoperbutylacetonicum* N1-4 ATCC13564 using either glucose or xylose as carbon source.

The spectrum of sugars present in the most promising feedstock for the ABE fermentation is quite assorted and the specific effects of each sugar on the fermentation performances have been recently addressed (Ezeji et al, 2007b; Raganati et al., 2012). Despite of this spectrum, a united model focusing on the effects of substrate on ABE fermentation has not been found in the literature. Therefore, this study present a kinetic dynamic model to investigate the effects of substrate on the ABE production by *C. acetobutylicum* DSM 792. The model has been implemented for several sugars: glucose, mannose, fructose, sucrose, lactose, xylose, and arabinose. The metabolic pathway specific of each sugar has been adopted: the Embden-Meyerhof-Parnas (EMP) pathway equations have been adopted for hexose and disaccharide sugars; the pentose phosphate (PP) pathway

equations have been adopted for pentose sugars. Two main issues have been addressed: 1) the dynamic behaviours of metabolites involved in ABE production, and 2) the inhibitory and activatory mechanism.

MATERIALS AND METHODS

Microorganism and media

Clostridium acetobutylicum DSM 792 was supplied by DSMZ (Braunschweig, Germany). Stock cultures were reactivated according to the DSMZ procedure. Reactivated cultures were stored at -80°C. The thawed cells were inoculated into 12 mL synthetic medium containing glucose (30 g/L) and Yeast Extract (5 g/L) in 15 mL Hungate tubes (pre-cultures). Cells were grown under anaerobic conditions for 48 h at 37 °C, then they were transferred into fermentation bottles. Each test was carried out in duplicate and the reported results are the mean values. The error was typiccally within 5 %.

The fermentation medium consisted of 5 g/L Yeast Extract, 2 g/L ammonium chloride (N-source) and 5 g/L $CaCO_3$ supplemented to P2 stock solution: buffer) 0.25 g/L KH_2PO_4, 0.25 g/L K_2HPO_4; mineral) 0.2 g/L $MgSO_4.7H_2O$, 0.01 g/L $MnSO_4.7H_2O$, 0.01 g/L $FeSO_4.7H_2O$ (15). The medium was sterilized in autoclave prior to the carbon source addition. Chemicals ($CaCO_3$, KH_2PO_4, K_2HPO_4, $MgSO_4.7H_2O$, $MnSO_4.7H_2O$, $FeSO_4.7H_2O$) and yeast extract were from Sigma Aldrich.

Sugars investigated were: glucose, mannose, fructose, sucrose, lactose, arabinose and xylose (Sigma Aldrich). Concentrated solutions of sugars were sterilized by filtration and supplemented to the autoclaved medium. The stock solution concentration of each investigated sugar was 300 g/L.

Analytical methods

pH was measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments). The analysis of culture samples withdrawn from cultures provided concentration in the liquid phase for biomass, sugars and metabolites, total organic carbon (TOC), and total nitrogen (TN). 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 OD_{600} = 0.4 g_{DM}/L (Napoli et al., 2010).

The concentration of soluble species was measured in the liquid phase after cell harvesting by centrifugation. Sugar concentration was measured by high performance liquid chromatography (HPLC) using an Agilent 1100 system (Palo Alto, CA). 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. Deionized water was used as mobile phase at a flow rate of 0.6 mL/min. Metabolite concentrations were measured by means of a GC apparatus equipped with a FID, and outfitted with a capillary column poraplot Q (25 m x 0.32 mm) was used. Internal standard (hexanoic acid) was adopted to assess acids and alcohols and their concentrations. The TOC/TN was measured with a Shimadzu TOC 5000A analyzer.

Operating conditions and procedures

Pyrex screw capped bottles (100 mL) containing 75 mL medium were used as fermenters. All tests were carried out: at rest, at 37 °C, without pH control. The medium was inoculated with 6.25 % (v/v) suspension of active growing pre-cultures. The initial cell concentration was about 1.5 mM. 3 mL of cultures were sampled

periodically for cell/metabolites characterization.

The initial concentration of the investigated sugar in each batch test was set at 60 g/L.

THEORETICAL FRAMEWORK

Model

The model was developed by using the biochemical network simulator software COPASI (Hoops et al., 2006). COPASI supports the development and the analyses of a reaction network. Moreover, it reports approximate velocity function of enzyme kinetics.

A kinetic simulation model (Proposed Model) of metabolic pathway has been developed addressing substrate utilization rate, production rate, and cell growth rate. The simulation model has been based on the metabolic pathways of *C. acetobutylicum* (Shinto et al., 2007, 2008). The pathways reported in Fig. 1A ($Model_{Hex/Disacc}$) have been assumed when glucose, mannose, fructose, sucrose, lactose are the carbon source. The pathways reported in Fig. 1B ($Model_{Pent}$) have been assumed when,xylose and arabinose are the carbon source. Glucose, fructose, mannose, sucrose and lactose are metabolized via the EMP pathway, and xylose and arabinose are metabolized via the PP pathway (Shinto et al., 2007, 2008). Details on the models are listed hereinafter.

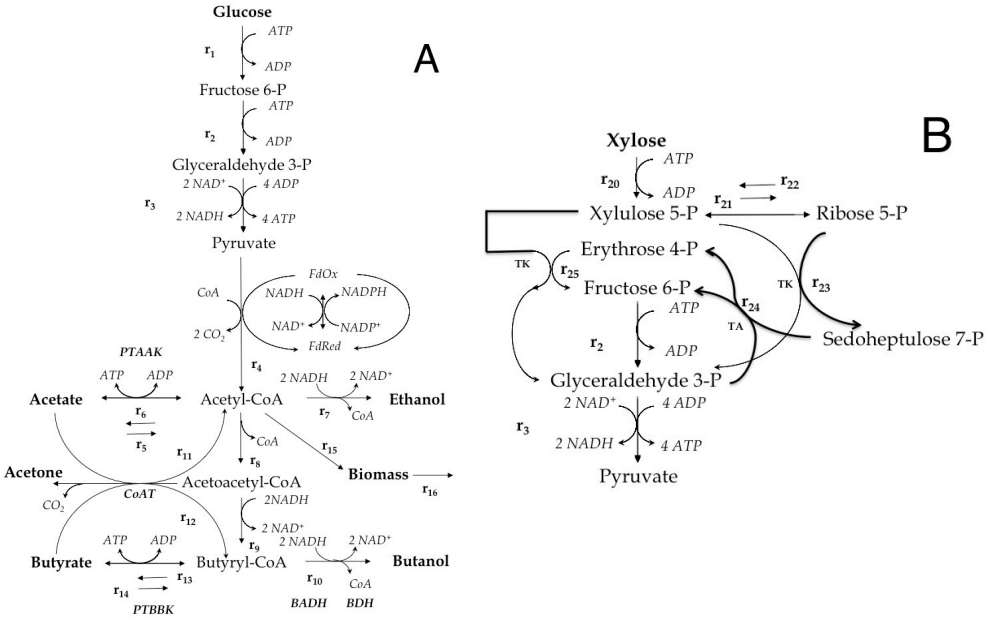

Fig. 1: A) Metabolic pathways of *C. acetobutylicum*. Carbone source: glucose, mannose, fructose, sucrose, lactose. Enzymes (bold style): PTA, phosphotransacetylase; AK, acetate kinase; CoAT, CoA transferase; PTB, phosphotransbutyrylase; BK, butyrate kinase; BADH, butyraldehyde dehydrogenase; BDH, butanol dehydrogenase. **B)** Metabolic PP pathways of *C. acetobutylicum*. Carbone source: xylose, arabinose. Enzymes (bold style): TA, transaldolase; TK, transketolase.

$Model_{Hex/Disacc}$ - the single steps of metabolic pathway described in Fig. 1A - hexoses and disaccharide sugars - are reported in Table 1 as reaction r_1 through r_{16}. $Model_{Pent}$ – the first steps of xylose and arabinose metabolism are reported in Table 1 as reaction r_{20} through r_{25}. Provided that G3P is produced, reaction r_2 through r_{16} hold also for the conversion of xylose and arabinose.

Main hyphotesis of the model proposed by Shinto et al. (2007, 2008) are hereinafter summarised:

- a Michaelis–Menten type kinetics characterized by butanol non-competitive inhibition is assumed for the rate equation of cell growth r^*_{15};.
- according to the reaction (1.16), the death rate of the cell - r_{16} – is assumed as a first order kinetics in the biomass concentration;
- the kinetic rates of reactions (1.1) and (1.20) – r^*_1 and r^*_{20}, respectively - is assumed by combining the substrate-sugar inhibition and the butanol-uncompetitive inhibition behaviour, according to the known effects of inhibization by substrate and solvent on substrate conversion rate (Jones and Woods, 1986);
- the kinetic rate of reaction (1.10) – r^*_{10} - is assumed by combining the butyrate activation and the butanol-uncompetitive inhibition behaviour, according to the enhacment effects of butyrate on the butanol production rate reported by (Tashiro et al., 2004).

Assumptions adopted in the present model are now on order. Kinetic relationships proposed in the present model are reported without the upscript "*".

- The kinetic expressions proposed by Shinto et al. (2007, 2008) for the sugar uptake (r^*_1, r^*_{20}), the butanol production (r^*_{10}), and the cell growth (r^*_{15}) are characterized by a gradually decrease of the rate to zero when butanol concentration approaches infinite. However, this behaviour does not fit the metabolism of C. acetobutylicum that is characterized by full inhibition as the concentration of inhibitor metabolites approaches a critical value (Zeng et al., 1994; Napoli et al., 2011, 2012). Moreover, the rate of the cell death (1.16) is activated by butanol (Gheshlagi et al., 2009). Accoording to the reported observations, the present model (Proposed Model) includes a modified set of reaction rates to substitute the uptake rates r^*_1 and r^*_{20}, the butanol production rate r^*_{10}, the the growth rate r^*_{15}, the cell death rate r^*_{16}. Table 1 reports the proposed reactation rate as: r_1 and r_{20}, complete inhibition as butanol concentration approaches the critical value B_{MAX} has been included; r_{15}, an interactive multiproduct-inhibited model has been adopted for the cell growth kinetics; r_{16}, a specific butanol activation expression has been included.
- The acetoacetyl-CoA transferase (CoAT) is characterized by a broad carboxylic acid specificity and it can catalyse the transfer of CoA to either acetate and butyrate (Shinto et al., 2007; Boynton et al., 1994). According to this observation, a specific expression of the reaction rate of CoAT have been proposed for each substrate in agreement with the random bi bi model (Gheshlaghi et al., 2009): r_{11} and r_{12}.
- The reaction rate equations of TK and TA consisted of random bi–bi mechanisms (Eqs. (1.20)–(1.22)).
- Several metabolic reactions related in ABE production require ATP or NADH (Fig. 1). It is expected that these reactions can not proceed if energy source depletion occurs - e.g. sugar exhaustion – and an on–off mechanism has been adopted. A switch-factor F has been introduced and it may assume value 1 or 0 depending on the substrate concentration in the broth. According to the observation of Okamoto et al. (1988), the threshold value of substrate concentration at which F switchs to 0 can is set larger than zero: F is set at 1 for substrate concentration larger than 1.00 mM. The switch-factor has been adopted for the rate of reactions that include ATP, ADP, NADH, and NAD+ among the susbstrtae: $r_1 – r_7$, r_9, r_{10}, r_{13}, r_{14} and r_7.

Assessment of the model parameters

Main assumptions adopted to assess model parameters are reported

hereinafter.

- The maximum reaction rate of a reaction step depends on the sugar because v_{max} depends on the enzyme concentration;
- The "affinity" constants - K_{aj}, K_{iij}, K_{isj}, K_{mj}, K_{ms16} - do not depend on the sugar because they depend on the enzyme responsible of the reaction step but not on its concentration.
- Parameters of the sugar uptake reactions (r_1 and r_{20}) have been assessed for each sugar. Indeed, Servinsky et al. (2010) reported that *C. acetobutylicum* has sugar-specific mechanisms for the transcriptional regulation of transport and metabolism genes. In particular, *C. acetobutylicum* utilizes: a) symporters and ATP-binding cassette (ABC) transporters for the uptake of pentose sugars; b) phosphotransferase system (PTS) transporters and a gluconate - H+ (GntP) transporter – for the uptake of disaccharides and hexoses. Moreover, the transcription of some transporter genes was found to be induced by specific sugars. Sugar-specific transport roles are suggested for various transporters of the PTS and of the ABC superfamily (Servinsky et al., 2010).

The assessment of the model parameters has been carried out according to the following procedure.

i. The proposed model has been adopted with reference to data measured during the glucose fermentation tests. The values of the kinetic parameters have been estimated to fit the experimental data of batch culture of *C. acetobutylicum* with the initial glucose concentration of 330 mM;
ii. The kinetic parameters characteristic of the hexoses (except glucose) and disaccharides (mannose, fructose, sucrose, lactose) have been assessed. Except for K_{aj}, K_{iij}, K_{isj}, K_{mj}, K_{ms16} (i and j ranging between 2 and 16), the maximum reaction rate v_{maxj} of each reaction step and the value of B_{MAXj}, $Acet_{MAX}$, $Butyr_{MAX}$, A_{MAX}, E_{MAX}, n_{BJ}, n_{Acet}, n_{Butyr}, n_A, and n_E have been assessed. The parameters of the reactoiion rate r_1 have also been estimated for each sugar.
iii. The kinetic parameters characteristic of the xylose PP pathway (r_{20}-r_{25}) have been assessed. Except for K_{aj}, K_{iij}, K_{isj}, K_{mj}, K_{ms16} (i and j ranging between 2 and 16), the maximum reaction rate v_{maxj} of each reaction step and the value of B_{MAXj}, $Acet_{MAX}$, $Butyr_{MAX}$, A_{MAX}, E_{MAX}, n_{BJ}, n_{Acet}, n_{Butyr}, n_A, and n_E have been assessed.
iv. The kinetic parameters characteristic of the arabinose pathway have been assessed: parameters of the reaction rate r_{20}, v_{maxj} of each reaction step, and B_{MAXj}, $Acet_{MAX}$, $Butyr_{MAX}$, A_{MAX}, E_{MAX}, n_{BJ}, n_{Acet}, n_{Butyr}, n_A, and n_E. Parameters not listed have been set at the values assessed for xylose pathway.

The soundeness of the model has been tested according two procedures:

- the assessment of the average squared correlation coefficients (r^2) between the simulation results and the experimental data.
- the comparison of the results of the presenet model with those reported by Shinto et al. (2007-2008).

RESULTS AND DISCUSSION

Batch cultures

Batch cultures have been carried out at initial sugar concentrations of 335 mM for hexose sugars (glucose, mannose, fructose), 167 mM for disaccharide sugars (sucrose, lactose) and 400 mM for pentose sugars (xylose, arabinose). Time

resolved concentration of sugar and metabolities have been measured. Data reported in Fig. 2 highlight that solvent production is sugar specific: glucose fermentation was characterized by the highest butanol concentration (169 mM).

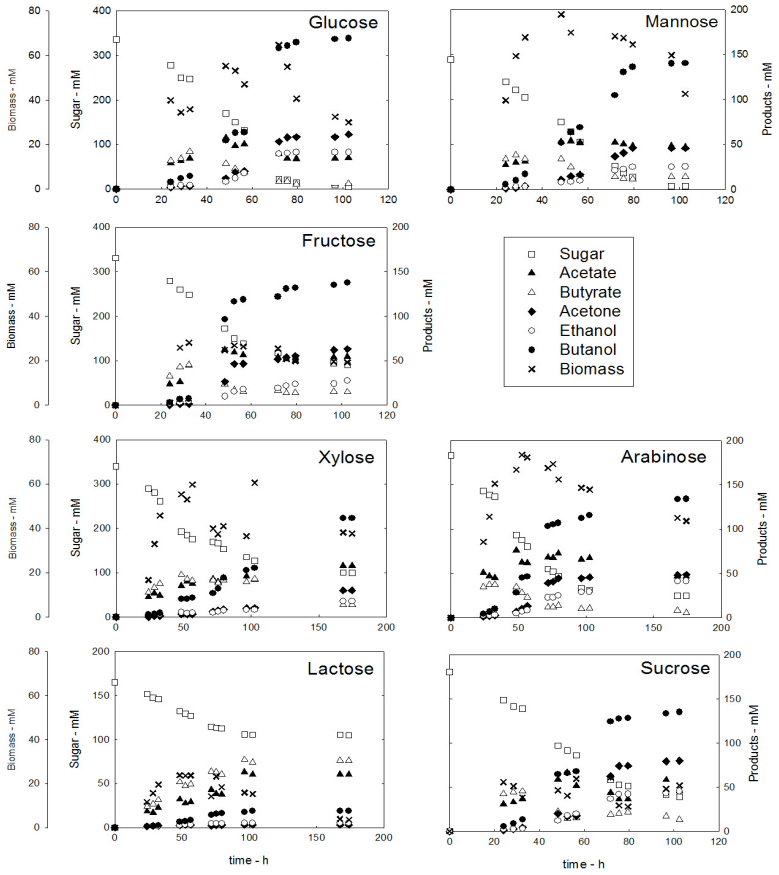

Fig. 2: Time-resolved concentration of target metabolites measured during batch cultures of glucose, mannose, fructose, sucrose, lactose, arabinose, and xylose.

Performances of mannose, fructose, and sucrose fermentations were slightly lower (butanol concentration 140, 134, and 135 mM, respectively); performances of arabinose and xylose fermentations decreased (116, and 112 mM, respectively); lactose fermentation was characterized by the lowest final butanol concentration (19 mM).

Effect of inhibition and activation terms

Data reported in Fig. 2 have been processed according the present model to assess the kinetic parameters of the metabolic pathways of *C. acetobutylicum* for hexoses/disaccharide (Fig. 1A) and pentoses (Fig. 1B). The procedure reported in section "*Assessment of the model parameters*" has been adopted.

The kinetic parameters assessed by processing the experimental data measured during the glucose fermentation test are reported in Table 2. Figure 3 reports the experimental data and the results of the plots from both present model simulation and Shinto's simulation (Shinto et al., 2007, 2008). The agreement of the present model with the experimental data is very satisfactory. The average correlation coefficient (r^2) reported in table 4 highlights that the present model is characterized by a higher likelihood (r^2=0.894) than that calculated for the Shinto's simulation (0.855).

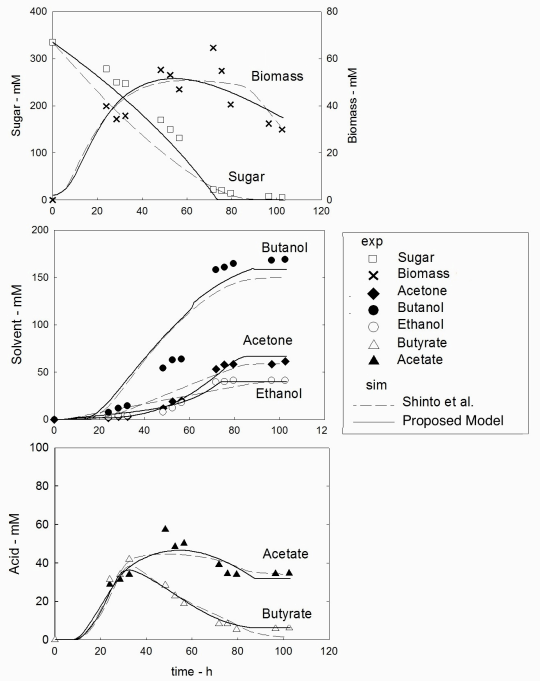

Fig. 3: Time-resolved concentration of glucose, biomass and metabolites. Experimental data vs. simulation results.

Table 3 reports kinetic parameters of the PP pathway (r_{20} through r_{25}) assessed by working out data measured during xylose. Figure 4 reports the experimental data and the results of the plots from both present model simulation and Shinto's simulation (Shinto et al., 2007, 2008). The agreement of the present model with the experimental data is very satisfactory. The average correlation coefficient (r^2) reported in table 4 highlights that the present model is characterized by a higher likelihood (r^2=0.89) than that calculated for the Shinto's simulation (0.83).

The kinetic parameters of the present model with reference to mannose, fructose, sucrose, lactose, and arabinose were assessed according to the procedure reported in section "*Assessment of the model parameters*". The simulations of the present model are ploted in Fig. 5 for the investigated sugar: the experimental dynamic behaviour of target metabolites have also been reported. Table 5 reports the kinetic parameters of sugar uptake (r_1 and r_{20}), butanol production (r_{10}), and cell growth (r_{15}) assessed for the fermentation of mannose, fructose, sucrose, lactose, arabinose, and xylose. The average r^2 for each sugar with reference to the present model and Shinto's simulation (Shinto et al., 2007, 2008) have been calculated and are reported in Table 4.

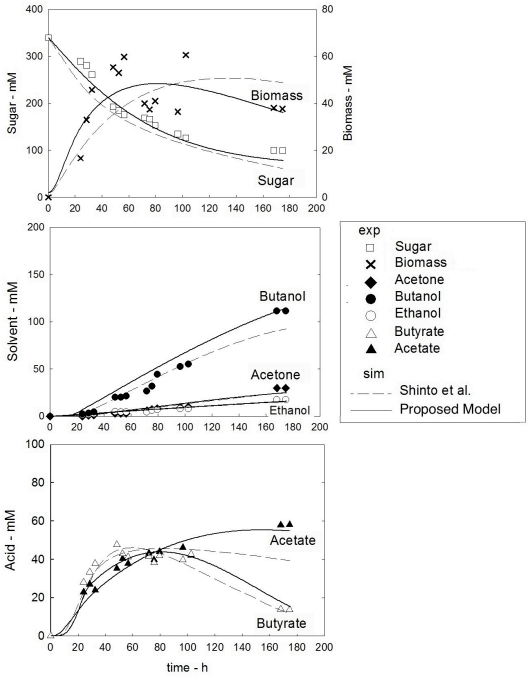

Fig. 4: Time-resolved concentration of xylose, biomass and metabolites. Experimental data vs. simulation results.

The analysis of the table suggests that r^2 for the simulations carried out by the present model increased with respect to that calculated for Shinto's simulation, whatever the tested sugar. The results confirmed that the structure of the present model improved the simulation results. In particular, main updates adopted in the present model with respect to the Shinto's model have been: complete inhibition term for butanol in the uptake kinetics (r_1 and r_{20}) and in the butanol production kinetics (r_{10}); an interactive multiproduct-inhibited model for the cell growth kinetics (r_{15}); a specific butanol activation expression for the cell death kinetics (r_{16}).

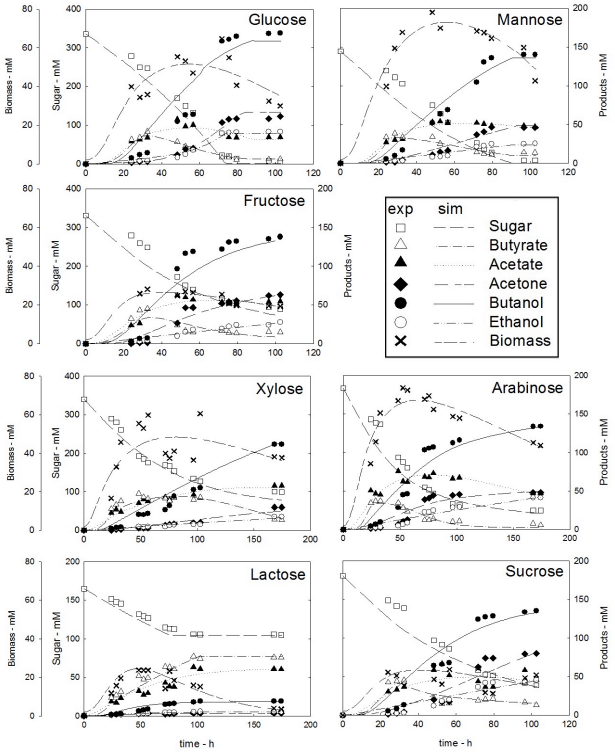

Fig. 5: Experimental time-course data and simulation results of target metabolites for glucose, mannose, fructose, sucrose, lactose, arabinose and xylose using the Proposed Model.

Results of the present model simulations have also been analysed to investigate the sensitivity of reaction steps with respect to the sugar. Figures 6 and 7 report the variation of the maximum reaction rate (V_{max}) of reaction steps with respect to the value assessed for glucose fermentation, for the hexose/disaccharide tested. Figure 8 reports the variation of V_{max} of reaction steps of the arabinose PP pathway with respect to the value assessed for xylose fermentation.

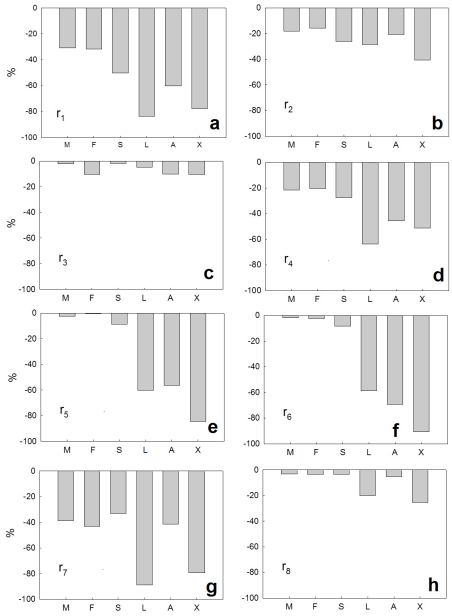

Fig. 6: Deviation of V_{max} of reaction r_1 through r_8 assessed for hexoses/disaccharides/pentoses with resepct to the value assessed for glucose as a function of the sugar.

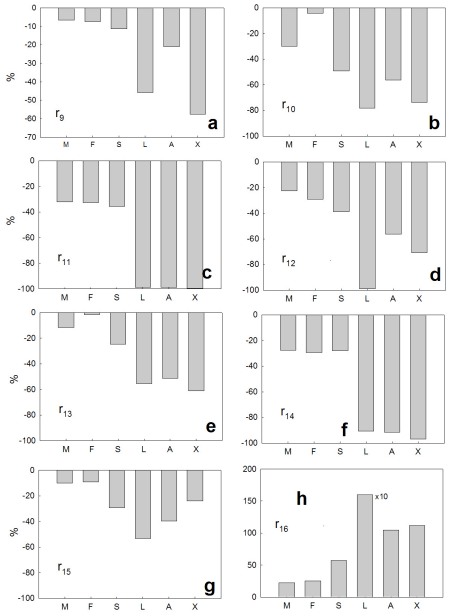

Fig. 7: Deviation of V_{max} of reaction r_9 through r_{16} assessed for hexoses/disaccharides/pentoses with resepct to the value assessed for glucose as a function of the sugar.

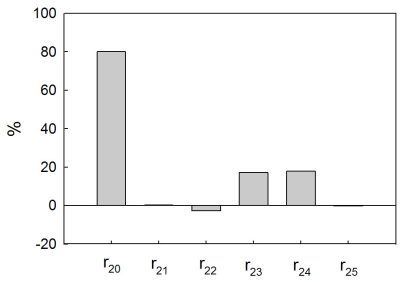

Fig. 8: Deviation of V_{max} of reaction r_{20} through r_{25} assessed for arabinose with resepct to the value assessed for xylose as a function of the sugar.

Main observations are reported hereinafter.

- Except for the cell death reaction (r_{16}), the V_{max} of each reaction step of hexose/disaccharides decreased with respect to the homologous step assessed for glucose fermentation. The V_{max16} assessed for glucose fermentation is smaller than the same parameter assessed for the hexose/disaccharides investigated.
- The reaction steps r_2, r_3, r_5, r_6, r_8, r_9, r_{13}, r_{15} for fructose and mannose fermentations are just barely affected by th sugar type: parameters differ less than 20% with respect to the homologous parameters assessed for glucose ferementation.
- For sucrose fermentation the reaction steps r_3, r_5, r_6, r_8, r_9 are just barely affected by th sugar type: parameters differ less than 20% with respect to the homologous parameters assessed for glucose ferementation.
- Except for r_3, the reaction steps of the *C. acetobutylicum* fermentation when lactose, arabinose, and xylose are adopted are remarkably affected by the sugar type: parameters differ more than 20% with respect to the homologous parameters assessed for glucose ferementation.
- The sugar uptake reactions - r_1 and r_{20} - reamarkably depends on the sugar type. The values of the parameters suggests that the *C. acetobutylicum* preference scale is: Glucose>Mannose/Fructose>Sucrose>Arabinose>Xylose>Lactose. This scale agrees with that reported in the literature regarding the “digeribility” of sugars (Ezeji et al, 2007b; Raganati et al., 2012)
- Except for the sugar uptake reaction r_{20}, the reactions of the PP pathway are not quite affected by the sugar. The kinetics of the reaction r_{20} remarkably depends on the sugar: the arabinose uptake is significantly faster with respect to xylose.

CONCLUSIONS

A kinetic simulation model has been proposed to describe the dynamic behaviour of metabolites in ABE fermentation by *Clostridium acetobutylicum*. The effects of the sugar on the kinetics has been investigated. The model has been addressed to assess parameters of reaction kinetics of hexoses/disaccharides pathways. The fermentation of glucose, mannose, fructose, sucrose, and lactose has been decribed according the Embden-Meyerhof-Parnas (EMP) pathway. The pentose phosphate (PP) pathway has been adopted for xylose and arabinose. The set of kinetics proposed by Shinto et al. (2007, 2008) has been updated to take into account in particular: i) the full inhibition behaviour of same reactions as the concentration of inhibitor metabolites approaches a critical value; ii) a specific butanol activation of the ceal death reaction; iii) an interactive multiproduct-inhibited model for

the cell growth.

The model gave satisfactory results for the tested sugars: the squared correlation coefficient (r^2) between experimental time-course of metabolites and calculated ones ranges between 0.87 and 0.925.

ACKNOWLEDGMENT

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NOMENCLATURE

[AACoA]	acetoacetyl-CoA concentration (mM)
ABE	acetone-butanol-ethanol
[Acet]	acetate concentration (mM)
[A]	acetone concentration (mM)
[ACoA]	acetyl-CoA concentration (mM)
Ar	Arabinose
[BCoA]	butyril-CoA concentration (mM)
[Biomass]	biomass concentration (mM)
[B]	butanol concentration (mM)
[Butyr]	butyrate concentration (mM)
[E]	ethanol concentration (mM)
F	switching factor of on-off mechanism
[F6P]	fructose 6-phosphate concentration (mM)
Fr	Fructose
G	Glucose
[G3P]	glyceraldehyde 3-phosphate concentration (mM)
j	number of the corresponding reaction in Fig. 1,2,3
[Pyr]	pyruvate concentration (mM)
K_{aj}	activation constant for activator (mM)
K_{iij}	inhibition constant for inhibitor (mM)
K_{isj}	inhibition constant for substrate (mM)
K_{mj}	concentration of metabolite where the rate is equal to half the value ov V_{max} (mM)
K_{msj}	specific activation constant (mM)
L	Lactose
M	Mannose
r_j	rate equation of metabolic reaction
V_{maxj}	maximum reaction rate (h^{-1})
S	Sucrose
[Sugar]	sugar concentration (mM)
YE	yeast extract
X	Xylose

Table 1: Reaction steps for the EMP and PP metabolic pathway and corresponding kinetics equations.

***** Kinetic relationships by Shinto et al. (2007, 2008).

Name	Reaction	Kinetics	Eq.
r_1	G/M/F -> F6P or S -> 2 F6P or L -> F6P + 2G3P	$r_1 = \frac{V_{\max 1}[S]}{K_{m1} + [S] + K_{m1}\left(\frac{[S]}{K_{is1}}\right)^2}\left(1 - \frac{[B]}{B_{MAX1}}\right)^{n_{B1}} \times F$	(1.1)
		$r_1^* = \frac{V_{\max 1}[Sugar]}{K_{m1} + K_{m1}([Sugar]/K_{is1})^2 + [Sugar](1 + [Butanol]/K_{i1})} \times F$	
r_2	F6P -> 2 G3P	$r_2 = \frac{V_{\max 2}[F6P]}{K_{m2} + [F6P]} \times F$	(1.2)
r_3	G3P -> Pyr	$r_3 = \frac{V_{\max 3}[G3P]}{K_{m3} + [G3P]} \times F$	(1.3)
r_4	Pyr -> ACoA	$r_4 = \frac{V_{\max 4}[Pyr]}{K_{m4} + [Pyr]} \times F$	(1.4)
r_5	ACoA -> Acetate	$r_5 = \frac{V_{\max 5}[ACoA]}{K_{m5} + [ACoA]} \times F$	(1.5)

r_6	Acetate -> ACoA	$r_6 = \frac{V_{max6}[Acet]}{K_{m6}+[Acet]} \times F$	(1.6)
r_7	ACoA -> E	$r_7 = \frac{V_{max7}[ACoA]}{K_{m7}+[ACoA]} \times F$	(1.7)
r_8	ACoA -> ½ AACoA	$r_8 = \frac{V_{max8}[ACoA]}{K_{m8}+[ACoA]}$	(1.8)
r_9	AACoA -> BCoA	$r_9 = \frac{V_{max9}[AACoA]}{K_{m9}+[AACoA]} \times F$	(1.9)
r_{10}	BCoA -> B	$r_{10} = \frac{V_{max10}[BCoA]}{K_{m10}(1+K_{a10}/[Butyr])+S\left(1-\frac{[B]}{B_{MAX10}}\right)^{n_{B10}}} \times F$ $r_{10}^{*} = \frac{V_{max10}[BCoA]}{K_{m10}(1+K_{a10}/[Butyrate])+[BCoA](1+[But\tan ol]/K_{ii10})} \times F$	(1.10)
r_{11}	Acet + AACoA -> A + ACoA	$r_{11} = V_{max11}\left(\frac{1}{1+K_{m11A}/[Acet]}\right)\times\left(\frac{1}{1+K_{m11B}/[AACoA]}\right)$	(1.11)
r_{12}	Butyr + AACoA -> A + BCoA	$r_{12} = V_{max12}\left(\frac{1}{1+K_{m12A}/[Butyr]}\right)\times\left(\frac{1}{1+K_{m12B}/[AACoA]}\right)$	(1.12)
r_{13}	BCoA -> Butyr	$r_{13} = \frac{V_{max13}[BCoA]}{K_{m13}+[BCoA]} \times F$	(1.13)
r_{14}	Butyr -> BCoA	$r_{14} = \frac{V_{max14}[Butyr]}{K_{m14}+[Butyr]} \times F$	(1.14)
r_{15}	ACoA -> Biomass	$r_{15} = \frac{V_{max15}[ACoA]}{K_{m15}+[ACoA]}\left(1-\frac{[Acet]}{Acet_{MAX}}\right)^{n_{Acetate}}\left(1-\frac{[Butyr]}{Butyr_{MAX}}\right)^{n_{Butyrate}}\left(1-\frac{[A]}{A_{MAX}}\right)^{n_A}\left(1-\frac{[E]}{E_{MAX}}\right)^{n_E}\left(1-\frac{[B]}{B_{MAX1}}\right)^{n_{B1}}$ $r_{15}^{*} = \frac{V_{max15}[ACoA]}{K_{m15}(1+[But\tan ol]/K_{ii15})+[ACoA](1+[But\tan ol]/K_{ii15})}$	(1.15)
r_{16}	Biomass -> Inactive Cells	$r_{16} = \frac{V_{max16}[Biomass][B]}{K_{ms16}\cdot K_{a16}+(K_{ms16}+[Biomass])\cdot[B]}$ $r_{16}^{*} = V_{max16}[Biomass]$	(1.16)
r_{20}	X/Ar -> X5P	$r_{20} = \frac{V_{max20}[S]}{K_{m20}+[S]+K_{m20}\left(\frac{[S]}{K_{is20}}\right)^2}\left(1-\frac{[B]}{B_{MAX20}}\right)^{n_{B20}} \times F$ $r_{20}^{*} = \frac{V_{max20}[Sugar]}{K_{m20}+K_{m20}([Sugar]/K_{is20})^2+[Sugar](1+[But\tan ol]/K_{ii20})} \times F$	(1.17)
r_{21}	X5P -> R5P	$r_{21} = \frac{V_{max21}[X5P]}{K_{m21}+[X5P]}$	(1.18)
r_{22}	R5P -> X5P	$r_{22} = \frac{V_{max22}[R5P]}{K_{m22}+[R5P]}$	(1.19)
r_{23}	R5P + X5P -> G3P + S7P	$r_{23} = V_{max23}\left(\frac{1}{1+K_{m23A}/[R5P]}\right)\times\left(\frac{1}{1+K_{m23B}/[X5P]}\right)$	(1.20)
r_{24}	G3P + S7P -> E4P + F6P	$r_{24} = V_{max24}\left(\frac{1}{1+K_{m24A}/[S7P]}\right)\times\left(\frac{1}{1+K_{m24B}/[G3P]}\right)$	(1.21)
r_{25}	E4P + X5P -> F6P + G3P	$r_{25} = V_{max25}\left(\frac{1}{1+K_{m25A}/[X5P]}\right)\times\left(\frac{1}{1+K_{m25B}/[E4P]}\right)$	(1.22)

***** Kinetic relationships by Shinto et al. (2007, 2008).

Table 1: Reaction steps of the EMP and PP metabolic pathways and associated kinetics.

Reactions	V_{max} (h^{-1})	K_m (mM)	K_{is} (mM)	K_{ii} (mM)	K_a (mM)	K_{ms} (mM)	K_{mA} (mM)	K_{mB} (mM)	A_{MAX} (mM)	B_{MAX} (mM)	E_{MAX} (mM)	$Acet_{MAX}$ (mM)	$Butyr_{MAX}$ (mM)	n_A	n_B	n_E	n_{Acet}	n_{Butyr}
r_1	6.79	0.07	7.75	299						220					0.71			
r_2	95	0.11																
r_3	305	212																
r_4	276	286																
r_5	8.20	7.20																
r_6	5.23	0.14																
r_7	0.48	0.09																
r_8	310	162																
r_9	248	9.56																
r_{10}	92	0.42		299	7.41					193					0.25			
r_{11}	6.67						0.001	276										
r_{12}	0.34						241	197										
r_{13}	722	0.05																
r_{14}	207	4.13																
r_{15}	2.43	0.0004		299					968	180	712	124.5	177	1	0.22	1	0.15	0.13
r_{16}	1.89				4.37	10.5												

Table 2: Kinetic parameters assessed by processing experimental data according to the present model. Carbon source – initial concentration: glucose, 335 mM.

Reactions	V_{max} (h^{-1})	K_m (mM)	K_{is} (mM)	K_{ii} (mM)	K_{mA} (mM)	K_{mB} (mM)	B_{MAX} (mM)	n_B
r_{20}	1.5	54	293	14.8			172	1.99
r_{21}	296	27						
r_{22}	218	43						
r_{23}	216				0.08	3.74		
r_{24}	205				5	22		
r_{25}	298				0.21	2.98		

Table 3: Kinetic parameters assessed by processing experimental data according to the present model. Carbon source-initial concentration: xylose, 400 mM.

r^2	Glucose	Mannose	Fructose	Sucrose	Lactose	Arabinose	Xylose
Shinto's simulation (*)	0.855	0.812	0.820	0.800	0.904	0.848	0.830
Present Model	0.894	0.887	0.870	0.880	0.925	0.904	0.890

*Shinto et al. (2007, 2008)

Table 4: Average squared correlation coefficients (r^2) between simulation results and experimental data.

Reaction	Parameters	Mannose	Fructose	Sucrose	Lactose	Arabinose	Xylose
r_1	V_{max} (h^{-1})	4.71	4.64	3.4	1.11	2.7	1.5
	K_m (mM)	0.1	0.04	0.49	40.8	46	54
	K_{is} (mM)	9	6	20	100	200	210
	B_{MAX}(mM)	199	199	199	153	186	171
	n_B	0.94	0.95	1.13	1.85	1.79	1.79
r_{10}	B_{MAX} (mM)	180	181	172	135	163	158
	n_B	0.46	0.47	0.58	1.62	0.62	0.64
r_{15}	$Acet_{MAX}$ (mM)	116	124	114	119	100	82
	$Butyr_{MAX}$ (mM)	179	150	141	141	137	127
	A_{MAX} (mM)	991	920	1096	972	875	810
	B_{MAX} (mM)	151	148	140	110	136	137
	E_{MAX} (mM)	722	760	765	725	689	661
	n_{Aacet}	0.72	0.79	0.93	1.39	0.91	1.21
	n_{Butyr}	0.26	0.28	0.37	0.87	0.72	0.87
	n_A	1	1	1	1	1	1
	n_B	0.76	0.73	0.83	1.93	1.18	1.85
	n_E	1	1	1	1	1	1

Table 5: Sugar uptake (r_1/r_{20}), butanol production (r_{10}) and cell growth (r_{15}) kinetic parameters for mannose, fructose, sucrose, lactose, arabinose and xylose.

5.2 MFA Of Clostridium Acetobutylicum Pathway: The Role Of Glucose And Xylose On The Acid Formation/Uptake

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ABSTRACT

ABE is typically produced during the second stage of batch fermentations of some *Clostridium* strains under selected operating conditions. The metabolic pathway of the ABE synthesis is quite complex and very few models are available in the literature to describe it. An alternative approach is the metabolic flux analysis (MFA). This contribution reports a MFA regarding *Clostridium acetobutylicum* DSM 792 fermentation adopting reference sugars (glucose and xylose) as carbon sources. The attention on these sugars is particular relevant because they are the main components of hydrolyzed lignocellulosic biomass. Results of batch fermentation tests carried out using glucose and xylose as carbon source have been adopted for flux assessments. The results have pointed out that the butyrate formation pathway plays a fundamental role both in the accumulation of butyrate and in butyrate uptake without acetone formation.

INTRODUCTION

The concerns regarding energy and environmental issues have revalued the interest in the use of biomass as a renewable energy source. According to this scenario, studies have bloomed in the scientific literature regarding the production of energy vectors from a wide spectrum of biomass. Solvent-producing clostridia could produce acetone, butanol, and ethanol (ABE) from several biomasses such as palm oil waste (Lee et al., 1995), agro-industrial waste(water)s (Raganati et al., 2013), and agricultural crops (Qureshi et al., 2001). The remarkable features of the butanol – e.g. hydrophobicity, high energy density, storage and transportation consistent with current structures – make this alcohol a potential substitute and/or supplement of gasoline (Cascone, 2008).

ABE is produced during the fermentations of some *Clostridium* strains - saccharolytic butyric acid-producing bacteria - under appropriate operating conditions (*C. saccharoperbutylacetonicum, C. acetobutylicum, C. beijerinckii, C. aurantibutyricum*). These strains are able to metabolize a great variety of substrates, pentoses, hexoses, mono-, di- and polysaccharides (Jones and Woods, 1986). Under batch conditions, the fermentation process of solvent-producing clostridium strains proceeds with the production of cells, hydrogen, carbon dioxide, acetic acid and butyric acid during the initial growth phase (acidogenesis) (Jones and Woods 1986). As the acid concentrations increase (pH decrease), the metabolism of cells shifts to solvent production (solventogenesis) and acidogenic cells – able to reproduce themselves - shift to the solventogenesis state with a morphological change (Jones and Woods, 1986). Two different physiological states must be taken into account for Clostridia: one for the acidogenic phase, and one for the

solventogenic phase. During the acidogenesis the acids production ensures high ATP and NADH yields. During the solventogenesis the active cells become endospores unable to reproduce themselves.

The spectrum of sugars present in the most promising feedstock for the ABE fermentation is quite large and studies available in the literature have pointed out that the fermentation performances depend on the sugar assortment (Ezeji and Blaschek, 2008; Raganati et al., 2012). Despite this large spectrum of feedstocks, models of the clostridia metabolism for the sugars are very few. Papoutsakis (1984) developed a stoichiometric model for the ABE production pathway from glucose. This model could be used to calculate or estimate the rates of reactions of the pathway in several ABE-producing clostridia. Desai et al. (1999) investigated the contribution of acid formation pathways in the metabolism of *Clostridium acetobutylicum* ATCC824T according to the metabolic flux analysis (MFA) and using glucose as carbon source.

A research program is active in Napoli regarding production of butanol by ABE fermentation (Napoli et al., 2010; Napoli et al, 2012; Raganati et al., 2013). In this study the MFA has been adopted to investigate the role of the main reaction steps of the *Clostridium acetobutylicum* metabolic pathway to convert reference sugars of hydrolyzed lignocellulosic biomass into butanol. Results of batch fermentation tests carried out using glucose and xylose as carbon source have been adopted for the flux assessment. The MFA has been implemented with reference to glucose and xylose as carbon source. The comparison of fluxes has suggested the relative role of each reaction step as a function of the carbon source investigated.

MATERIALS AND METHODS

Microorganism and medium

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Stock cultures were reactivated according to the DSMZ procedure. Reactivated cultures were stored at -80°C. The thawed cells were inoculated into 12 mL synthetic medium containing glucose (30 g/L) and Yeast Extract (YE) (5 g/L) in 15 mL Hungate tubes (pre-cultures). Cells were grown under anaerobic conditions for 48 h at 37 °C, then they were transferred into fermentation bottles. The fermentation medium consisted of 5 g/L YE and 5 g/L of $CaCO_3$ supplemented to P2 stock solution: buffer) 0.25 g/L KH_2PO_4, 0.25 g/L K_2HPO_4, 2 g/L ammonium chloride; mineral) 0.2 g/L $MgSO_4.7H_2O$, 0.01 g/L $MnSO_4.H_2O$, 0.01 g/L $FeSO_4.7H_2O$ (Napoli et al, 2011). The medium was sterilized in autoclave prior to the carbon addition. The carbon source (single sugar) was supplemented to the medium and sterilized by filtration. Two sugars – glucose and xylose - were investigated. The overall initial concentration of each sugar was set at 60 g/L.

Batch fermentation

Pyrex screw capped bottles (100 mL) containing 75 mL medium were used as fermenters. All experiments were carried out in fermenters at rest, at 37 °C, without pH control. The medium was inoculated with 6.25 $\%_v$ suspension of active growing pre-cultures. 3 mL of cultures were sampled periodically for cell/metabolites characterization.

Analytical procedures

pH was measured off-line in 1.5 mL samples by a pH-meter (Hanna Instruments). Analysis of culture samples was carried out after centrifugation at 10,000 rpm for 10 min. The liquid phase was characterized in terms of sugar and metabolite concentrations. Cell density was determined by measuring the absorbance at 600 nm (Cary-Varian mod. 50 scan UV-VIS spectrophotometer).

Calibration tests indicated that the optical density is proportional to *C. acetobutylicum* dry mass under the operating conditions tested, in particular 1 OD_{600} corresponded to 0.4 g_{DM}/L. Sugar concentration was determined by high performance liquid chromatography (HPLC) using an Agilent 1100 system (Palo Alto, CA). The sugars were separated on a 8 µm Hi-Plex H, 30 cm 7.7 mm at room temperature and detected with a refractive index detector. Deionized water was used as mobile phase at a flow rate of 0.6 mL/min. A GC apparatus equipped with a FID, and outfitted with a capillary column poraplot Q (25 m x 0.32 mm) was used.

METABOLIC FLUX ANALYSIS

According to the metabolic flux analysis (Papoutsakis, 1984), the assessment of in vivo fluxes has been carried out by processing the time-resolved concentration of substrates and products. The analysis has been based on the known metabolic pathway (Fig. 1) translated into a set of reactions, e.g. a set of linear equations. The reaction set regarding the glucose conversion is reported in Table 1 as Eq.s (T.1.1) through (T.1.13): reversible reactions are indicated with '+/-', irreversible reactions are indicated with '+' (Desai et al., 1999). The r_{PTAAK} and r_{PTBBK} refer to the reactions catalysed by the enzyme couples PTA-AK and PTB-BK, respectively.

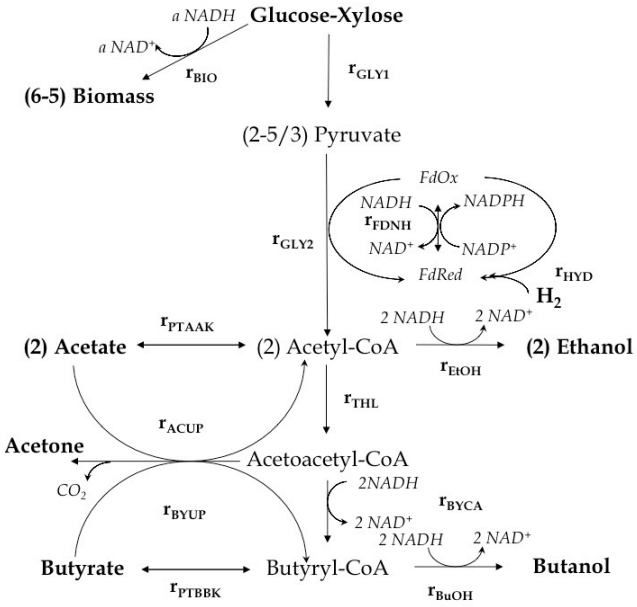

Fig.1*: C. acetobutylicum* metabolic pathway and relevant fluxes. The conversion between major carbon containing species is depicted (cofactors are not reported).

Table 1: Reaction step of *Clostridium acetobutylicum* metabolic pathway

Reaction Step	Flux	
$GLU \xrightarrow{-\frac{6\cdot 12}{\sigma_X \cdot Y_{XIATP}}ATP - aNADH} 6BIO \qquad a=\frac{6}{2}\left(\gamma_X - \gamma_{GLU}\right)$	+/-r_{BIO}	(T.1.1)
$GLU \longrightarrow 2PYR + 2ATP + 2NADH$	+r_{GLY1}	(T.1.2)
$PYR \longrightarrow ACoA + CO_2 + FdRed$	+r_{GLY2}	(T.1.3)
$ACoA + 2NADH \longrightarrow E$	+r_{EtOH}	(T.1.4)
$ACoA \longrightarrow AA + ATP$	+/-r_{PTAAK}	(T.1.5)
$2ACoA \longrightarrow AACoA$	+/-r_{THL}	(T.1.6)
$AACoA + AA \longrightarrow A + ACoA + CO_2$	+r_{ACUP}	(T.1.7)

$AACoA + AB \longrightarrow BCoA + A + CO_2$	$+r_{BYUP}$	(T.1.8)
$AACoA + 2NADH \longrightarrow BCoA$	$+/-r_{BYCA}$	(T.1.9)
$BCoA \longrightarrow AB + ATP$	$+/-r_{PTBBK}$	(T.1.10)
$BCoA + 2NADH \rightarrow B$	$+r_{BuOH}$	(T.1.11)
$FdRed \rightarrow H_2$	$+r_{HYD}$	(T.1.12)
$FdRed \rightarrow NADH$	$+/-r_{FDNH}$	(T.1.13)
$XYL \xrightarrow{-\frac{5\cdot 12}{\sigma_X \cdot Y_{X/ATP}}ATP - \frac{5}{2}(\gamma_X - \gamma_{XYL})NADH} 5BIO$	$+-r_{BIO}$	(T.1.14)
$XYL \longrightarrow \frac{5}{3}PYR + \frac{5}{3}ATP + \frac{5}{3}NADH$	$+r_{GLY1}$	(T.1.15)

Table 1*: Reaction step of Clostridium acetobutylicum metabolic pathway*

The MFA of xylose fermentation has been proposed by substituting the reaction steps (T.1.1) and (T.1.2) with (T.1.14) and (T.1.15). The reactions regarding ATP have not been considered in the present model. It has been assumed that the produced ATP is consumed by cell growth process and cell non-growth maintenance. The flux r_{BIO} has been adopted as a measure of both growth and lysis of cells and it is based on a dynamical shifting of the stoichiometric coefficients of glucose/xylose and NADH: i) for positive values of r_{BIO}, the coefficients reported in table 1 were used; for negative values of r_{BIO}, the coefficient of glucose/xylose and NADH were set at 0.

The stoichiometric matrix of the model was characterized by a singularity that prevented the assessment of a unique set of fluxes of the metabolic activity. The non linear constraint proposed by Desai et al. (1999) was adopted to solve the model equation set. The constraint relates the uptake fluxes of acetate (r_{ACUP}) and butyrate (rBYUP):

$$\frac{r_{BYUP}}{r_{ACUP}} = 0.315\frac{[butyrate]}{[acetate]} \quad (1)$$

The stoichiometric model of solventogenic clostridia has been restructured as a constrained minimization problem of the objective function Eq. (2):

$$\| A \cdot r - x \|^2 + \left(r_{BYUP}[acetate] - 0.315[butyrate]\right)^2 \quad (2)$$

where the first term of Eq. (2) is the sum of weighted squared residuals, and the second term is from the Eq. (1). A is the stoichiometric matrix, x the species accumulation vector, and r the pathway flux vector.

RESULTS AND DISCUSSION

Fig. 2 shows metabolite concentration measured during batch fermentations of *C. acetobutylicum* on both glucose and xylose as carbon source. As expected, acetate and butyrate were produced/accumulated at the beginning of the fermentation, and acetone, butanol and ethanol were produced/accumulated at a second stage. The A:B:E molar ratio was about 3:6:2 and 1:6:1 for glucose and xylose respectively.

The concentration time series were processed to asses the specific net rates (production/consumption) of acetate, butyrate, and acetone (Fig. 3 A-C) for both glucose and xylose fermentation. The specific net rates referred to the unit biomass concentration expressed as OD. The vertical dotted lines mark the transition to the solventogenesis phase. The analysis of the specific net rates referred to glucose (Fig. 3A) pointed out that: i) acetate and butyrate rates were maximum at the beginning of the fermentation and gradually decreased during the transition to the solventogenesis phase; ii) acetate and butyrate rates were lower than zero during the

solventogenesis phase to point out the uptake of both acids; iii) the rate of acetone increased at the solventogenesis threshold and it reached its maximum value late during the solventogenesis phase.

The rates reported in Fig. 3C were calculated for the fermentation tests carried out adopting xylose as carbon source (Fig. 2B). The main dynamics observed for the test carried out adopting glucose as carbon source are still observed. The main differences are: i) the butyrate specific net rate was definitively negative during the solventogenesis phase and the acetate uptake rate is nearby zero, that is only butyrate is definitively converted during solventogenesis; ii) the acetone production rate is quite low throughout the test.

It is worth to note that the specific net rate of acetone departs from zero before the acidogenesis phase ended. This observation confirms that measured uptake of acetate and butyrate are net values and acid formation rates are higher than the measured specific net values. The specific net rates do not accurately describe metabolic activity.

Metabolic flux analysis has been adopted to assess the un-observable patterns of the metabolic activity. The specific in vivo fluxes as estimated according to the MFA are reported in Fig.s 3 B and 3D. The rate r_{PTAAK} assessed for both glucose and xylose metabolisms decreased gradually throughout the fermentation and approached zero. The observed r_{PTAAK} vs. time profile is consistent with the acetate specific net rate vs. time profile. However, a fundamental difference may be noted between glucose and xylose fermentation as reported hereinafter.

Glucose – i) it has been assessed that the acetate is continuously produced (not consumed) according to the reaction (T.1.5) throughout the fermentation, indeed r_{PTAAK} is always positive during the fermentation (Fig. 3B); ii) it has been measured that the acetate is converted during the late solventogenesis phase (Fig. 3A). The analysis of results suggests that acetate uptake is via the acetone formation pathway, r_{ACUP}.

Xylose – i) it has been assessed that the acetate is continuously produced (not consumed) according the reaction (T.1.5) throughout the fermentation, indeed r_{PTAAK} is always positive during the fermentation (Fig. 3D); ii) the acetate conversion (r_{PTAAK}) vs time (Fig. 3D) looks like the acetate production rate. The analysis of results suggests that the acetone formation pathway, r_{ACUP}, is negligible.

The butyrate formation pathway appears to change during the different phases of the cultures. The r_{PTBBK} assessed for both carbon sources decreases gradually since the beginning of the fermentation tests and it is a proof of that the butyrate is produced according to the butyrate formation pathway. As the solventogenesis starts, the butyrate formation pathway acts to uptake the butyrate (negative values of r_{PTBBK}, Fig.s 3B and 3D). Although the reconversion of butyrate has been assessed for both carbon sources, this process is more marked in glucose fermentation than in xylose fermentation (Fig. 3B vs. Fig. 3D). As regards the role of the acetone formation pathway in the fate of the butyrate, the butyrate formation pathway appears to dominate the uptake with respect to the acetone formation pathway.

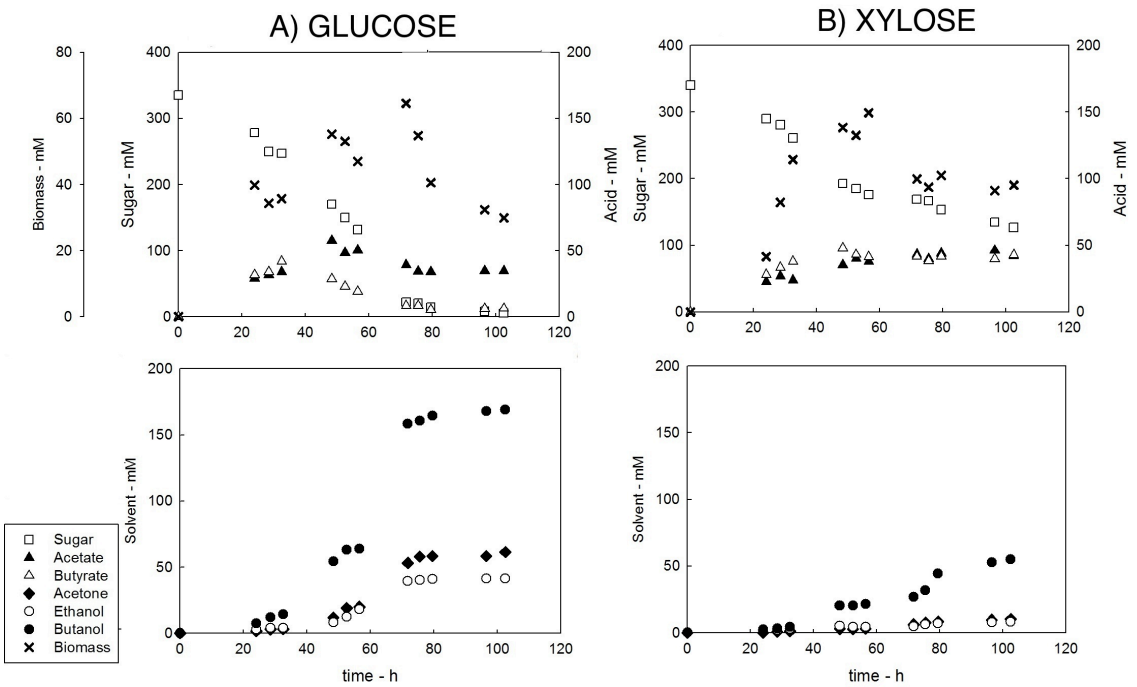

Fig.2*: Time resolved concentration of metabolities measured during C. acetobutylicum* batch fermentation.

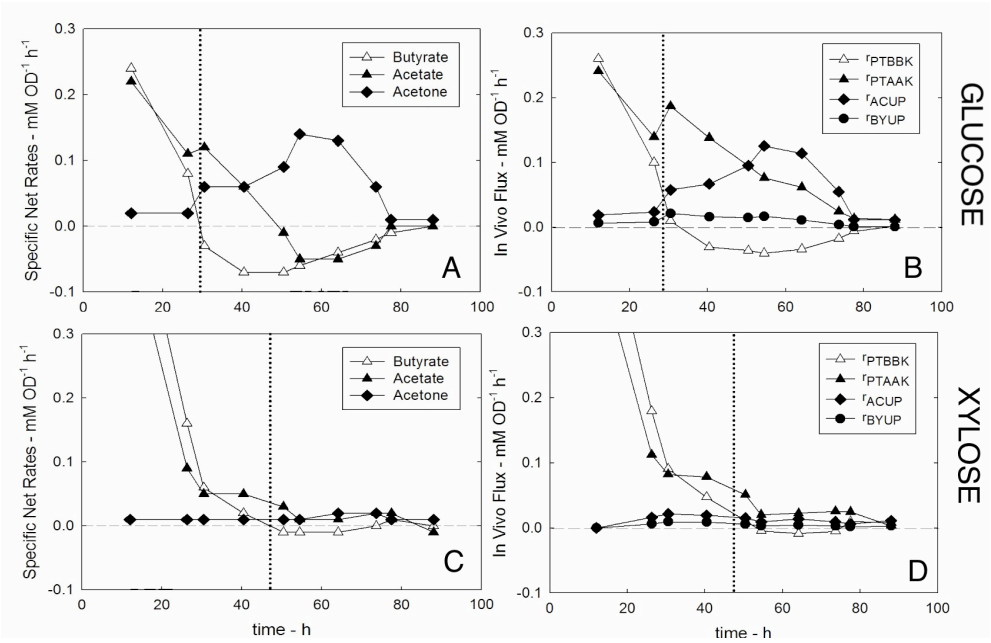

Fig.3*: A-C) Specific net rates of production (>0) or consumption (<0) of acetate, butyrate and acetone calculated by processing data reported in Fig. 2. B-D) Specific in vivo fluxes assessed by MFA. Dashed horizontal lines mark zero net rate or in vivo flux. Vertical dotted lines mark the transition to the solventogenesis.*

The analysis of reported results regarding the butyrate fate points out that PTB and BK are still active during solventogenesis and they are responsible of the butanol production at a low acetone/butanol ratio. This scenario is still more marked for xylose fermentation. The higher (absolute) values of r_{PTBBK} calculated for glucose fermentation under solventogenic phase with respect to those calculated for xylose fermentation would suggest that a high butyril-CoA production is expected for glucose fermentation. The higher values of butyril-CoA concentration are in

agreement with the higher butanol concentration measured at the end of the glucose fermentation (Fig. 2).

FINAL REMARKS

Clostridium acetobutylicum fermentation of both glucose and xylose has been successfully carried out to produce butanol. Results have been interpreted according the metabolic flux analysis (MFA). The analysis of the MFA has pointed out the roles played by acid formation enzymes in the complex primary metabolism of solventogenic clostridia. In particular, the effects of the carbon source on the relevance of each step of the acid formation/uptake pathway have been highlighted. The butyrate formation pathway plays a fundamental role both in the production of butyrate and in butyrate uptake without acetone formation.

6 REACTOR SYSTEM DEVELOPMENT

The activity has regarded the design, set-up and operation of biofilm fixed bed reactors for the butanol production. Unsupplemented cheese whey has been adopted as renewable feedstock. Operating conditions of the continuous tests have been selected to maximize the butanol production and butanol selectivity.

The success of operation of the Packed Bed Reactor (PBR) has fuelled the development of an innovative continuous biofilm reactor configuration: "carosello" of packed bed biofilm reactors connected in series. The reactor system has been equipped with a device to switch the feeding at the reactor according to a pre-set sequence. A mathematical model to support the bioreactor system design has been proposed.

6.1 Butanol Production By Bioconversion Of Cheese Whey In A Continuous Packed Bed Reactor

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ABSTRACT

Butanol production by *Clostridium acetobutylicum* DSM 792 fermentation was investigated. Unsupplemented cheese whey was adopted as renewable feedstock. The conversion was successfully carried out in a biofilm packed bed reactor (PBR) for more than three months.

The PBR was a 4 cm ID, 16 cm high glass tube with a 8 cm bed of 3 mm Tygon rings, as carriers. It was operated at the dilution rate between 0.4 h^{-1} and 0.94 h^{-1},
The cheese whey conversion process was characterized in terms of metabolites production (butanol included), lactose conversion and of mass of biofilm. Under optimized conditions it resulted: butanol productivity 2.66 g/Lh, butanol concentration 4.93 g/L, butanol yield 0.26 g/g, butanol selectivity of the overall solvents production 82%$_w$.

Keywords: cheese whey, butanol, biofilm fixed bed reactor, ABE

INTRODUCTION

The socio-economic scenario characteristic of the beginning of the third millennium revives the interest in strategy for bioconversion of industrial wastewaters in biofuels and bulk chemicals. In this context, the Acetone-Butanol-Ethanol (ABE) fermentation is receiving renewed interest as a way to upgrade renewable resources into valuable base chemicals and liquid fuels (Ezeji et al, 2007a; Dürre, 2008; Friedl, 2012). Indeed, butanol offers several advantages over ethanol for gasoline-alcohol blending because of its high energy content, low miscibility with water, and low volatility (Bohlmann, 2007; Cascone, 2008). In addition, butanol can replace gasoline without any modification of the current vehicle and engine technologies.

ABE is typically produced during the later stage of batch fermentations of some *Clostridium* strains - saccharolytic butyric acid-producing bacteria - under appropriate

operating conditions (*Clostridium saccharoperbutylacetonicum, C. acetobutylicum, C. beijerinckii, C. aurantibutyricum*). The strains are able to metabolize a great deal of substrates: pentoses, hexoses, mono-, di- and polysaccharides (Flickinger and Drew, 1999). Under batch conditions, the fermentation process of solvent-producing clostridia proceeds with the production of cells, hydrogen, carbon dioxide, acetic acid and butyric acid during the initial growth phase (acidogenesis) (Jones and Woods, 1986). As the acid concentration increases (pH decreases), the metabolism of cells shifts to solvent production (solventogenesis) and acidogenic cells – able to reproduce themselves - shift to the solventogenesis state with a morphological change (Jones and Woods, 1986). Under solventogenesis, the active cells become endospores unable to reproduce themselves. Accordingly, two different physiological states must be taken into account for clostridia: one for the acidonegenic phase, and one for the solventogenic phase.

Despite the aforesaid remarkable advantages of butanol as a bioproduct, its industrial production via fermentation is not developed for several issues: feedstock cost and availability, low value of the yield and productivity, low concentration of butanol in the broth (as a consequence of the product-inhibition feature of the ABE fermentation), degeneration of butanol-producing strains (Kumar and Gayen, 2011). Moreover, the low butanol concentration in fermentation broth makes its recovery and concentration quite complex (Liu and Fan, 2004; Ezeji et al., 2007b; Napoli et al., 2012a).

Performance enhancements were proved by continuous ABE production in reactors with immobilised *clostridium* strains (Lee et al., 2008b; Qureshi et al., 2000; Napoli et al., 2010). Indeed, the high cell density typical of this reactor typology improves butanol yield and butanol recovery. The continuous bioconversion in immobilized cell reactors is characterized by several advantages with respect to batch cultures, typically adopted for butanol production via fermentation route (Qureshi et al., 2000b). Main advantages are related to the high cell concentrations and to the reactor operability at high dilution rate without cell washout (Welsh et al. 1987). Moreover, cell support may often be reused (Krouwel et al. 1980).

As regards feedstocks, abundance and un-competitiveness with food sources are prerequisites of potential substrates. Typically, these requisites are fulfilled by lignocellulosic biomass and wastewater streams. The former category of feedstocks may be both purposely cultivated and agro-industrial by-products. The latter category includes cheese-whey, wastewaters. The potential of the cheese-whey as feedstock for butanol production has been pointed out by several authors (Qureshi and Maddox, 1987; Siso, 1996; Foda et al., 2010). Tests typically regarded batch fermentations (Foda et al., 2010) and continuous bioreactors fed with whey permeate supplemented with yeast extract (Qureshi and Maddox, 1987).

The aim of this contribution is to investigate the feasibility of bio-butanol production by continuous conversion of cheese whey, a dairy industry wastewater characterized by high lactose and protein content. The anaerobic solventogenic bacterium *Clostridium acetobutylicum* DSM 792 was adopted for the fermentation processes. The conversion was carried out in a packed bed reactor (PBR) of Tygon rings as biofilm carriers. The process was characterized in terms of butanol production rate, butanol selectivity and butanol yield as a function of the dilution rate.

MATERIALS AND METHODS

Microorganism

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Stock cultures were reactivated according to the DSMZ procedure. Reactivated cultures were stored at -80°C. The thawed cells were inoculated in 15 mL Hungate tubes containing 12 mL synthetic medium: 20 g/L lactose, 5 g/L Yeast Extract (YE). Cells were grown under anaerobic conditions for 48 h at 37 °C. Then pre-cultures were transferred into fermentation bottles.

Medium

Synthetic medium

The synthetic medium consisted of 20 g/L lactose and 5 g/L YE supplemented with P2 stock solution: buffer) 0.5 g/L KH_2PO_4, 0.5 g/L K_2HPO_4, 2 g/L ammonium chloride; mineral) 0.2 g/L $MgSO_4.7H_2O$, 0.01 g/L $MnSO_4.H_2O$, 0.01 g/L $FeSO_4.7H_2O$ (Qureshi and Blaschek, 2000). The lactose-YE solution was sterilized in autoclave (20 min at 120 °C).

Cheese whey powder

The cheese whey powder (CWP) was from the Sierolat - an Italian firm – and its composition is reported in Table 1.

	w%
Lactose	69
Proteins	12.5
Ashes	7.5
Lactic Acid	4
Moisture	2.5
Fats	2.5
Galactose	2

Table 1: Composition of the cheese whey powder

The CWP was solubilized in deionized water (concentration 40 g/L) and pretreated according to methods reported in Table 2.

The continuous biofilm reactor was fed with a pretreated solution of CWP characterized by 28 g/L lactose concentration.

Apparatus and Operating Conditions

All experiments were carried out at 37 °C.

Batch Fermentation

Pyrex screw capped bottles (100 mL) containing 75 mL medium were used as fermenters. Tests were carried out in fermenters at rest without pH control. The medium was inoculated with 6.25 % (v/v) suspension of active growing pre-cultures. 3 mL of cultures were sampled periodically for the concentration measurement of lactose and metabolites.

Continuous bioreactor

The apparatus adopted for the cheese whey fermentation consisted of a fixed bed reactor, liquid pumps, a heating apparatus, a device for the pH control, and on-line diagnostics (Fig. 1).

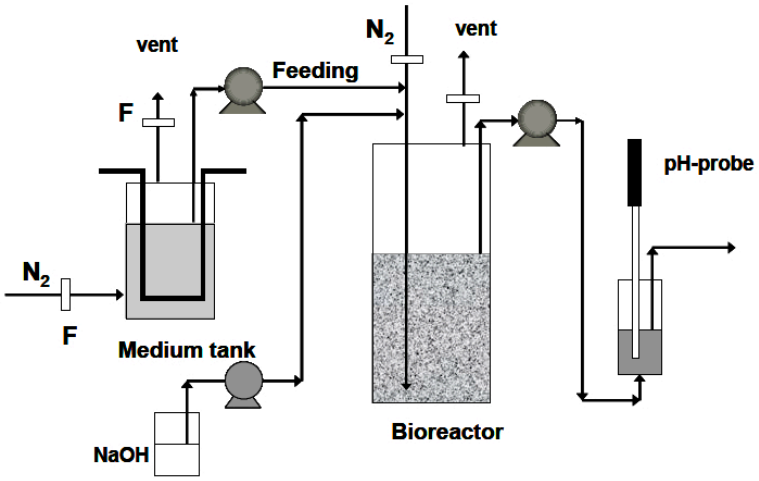

Fig.1: Outline of the apparatus adopted for continuous test equipped with a packed bed biofilm reactor. F) gas sterilization filter.

The fixed bed was at the bottom of a 200 mL glass lined pipe (4 cm ID, 16 cm high) jacketed for the heat exchange. The liquid phase volume in the reactor, or reaction volume, was modified by changing the level of the overflow duct. Nitrogen was sparged at the reactor bottom to support anaerobic conditions. The device for the pH control consisted of a pH-meter, a peristaltic pump, a vessel with NaOH 0.3 M solution, and a controller. The reactor with the carriers was sterilized in autoclave at 121 °C for 20 min. The gas stream was sterilized by filtration. The sterile medium was fed at the bottom of reactor by means of a peristaltic pump.

Tygon rings (3/1 mm OD/ID) were selected as biofilm carriers according to previous investigation (Napoli et al., 2010).

Analytical Procedures

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

Analysis of culture samples was carried out after centrifugation at 10,000 rpm for 10 min. The liquid phase was characterized in terms of lactose and metabolite concentrations, total organic carbon (TOC), and total nitrogen (TN). Lactose concentration was determined by high performance liquid chromatography (HPLC) using an Agilent 1100 system (Palo Alto, CA). Lactose was separated at room temperature by means of a 8 µm Hi-Plex H, 30 cm 7.7 mm and detected with a refractive index detector. Deionized water was used as mobile phase at 0.6 mL/min flow rate. A GC apparatus Agilent 6890 series (Palo Alto, CA) equipped with a capillary column poraplot Q (25 m x 0.32 mm) and a FID was used for the metabolite analysis. Hexanoic acid was adopted as internal standard to analyse acids and alcohols and to assess their concentrations. A Shimadzu TOC 5000A analyzer was adopted to measure TOC/TN concentration.

Procedures

300 microliter of stock culture was transferred in 4 15-mL Hungate tubes containing the culture media (20 g/L of lactose). The cultures were incubated for 1 days under batchwise anaerobic sterile conditions, then 40 mL of active culture were inoculated in the reactor.

The biofilm in the fixed bed reactor was grown adopting lactose-based medium at the beginning of the test and setting the pH compatibly with acidogenic phase. The

feeding was switched to CWP-based medium and after a pre-set time the pH was definitively decreased for solvent production.

Tests aimed at the butanol production were carried out with the biofilm PBR operated at selected conditions. The dilution rate – ratio between the feeding volumetric flow rate and the volume of the fixed bed and – ranged between 0.54 and 0.94 1/h. Each steady state was characterized in terms of metabolites and lactose concentration. The biomass inside the reactor was measured at the end of the test program by sacrificing the reactor (Qureshi et al. 1987). The mass of biofilm in the reactor was assessed at the end of the run in agreement with the following procedure: (i) the dry carrier was weighted before filling the reactor; (ii) at the end of the test, the reactor was rinsed with sterile water to remove lactose and metabolites; (iii) the supports with the biomass were harvested and dried at 40 °C for 1 day; (iv) the dried mass of the biomass and supports was weighted. The dried mass of the biofilm in the reactor was assessed as the difference between the weight of the supports-biofilm and the supports.

Lactose and metabolite concentrations were worked out to assess the data reported hereinafter. In particular, it was assumed that the feeding was aseptic and free of metabolites and that the gas stripping of metabolites was negligible. Lactose conversion (ξ_L), lactose-to-"i-species" fractional yield coefficient ($Y_{i/L}$), butanol productivity (W_B), ABE productivity (W_{ABE}), butanol selectivity (Φ) were assessed according with the relationships:

$$\xi_L = \frac{(L^{IN} - L^{OUT})}{L^{OUT}} \quad (1)$$

$$Y_{i/L} = \frac{i^{OUT}}{(L^{IN} - L^{OUT})} \quad (2)$$

$$W_B = D \cdot B^{OUT} \quad (3)$$

$$W_{ABE} = D \cdot ABE^{OUT} \quad (4)$$

$$\Phi = \frac{D \cdot B^{OUT}}{D \cdot (A^{OUT} + B^{OUT} + E^{OUT})} \quad (5)$$

where L, A, B and E are the concentration of lactose, acetone, butanol and ethanol, respectively, measured in the feeding (suffix IN) and in the effluent (suffix OUT). The performances were assessed working out the concentration of lactose and metabolites, provided that the steady-state was stabilized for at least 10 times the reactors mean residence time (1/D).

THEORETICAL FRAMEWORK

Performances of the packed bed reactor were interpreted taking into account: i) coexistence in the biofilm of acidogenic cells and solventogenic cells , and ii) reactions responsible of acids production/conversion.

Figure 2 shows a sketch of a biofilm section.

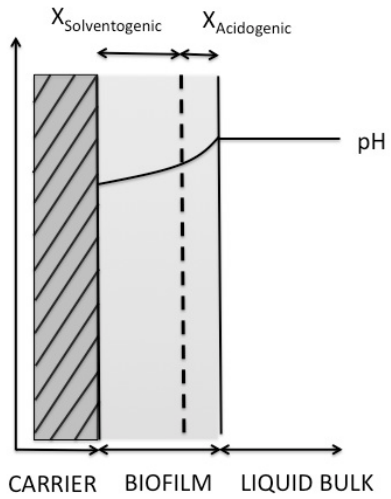

Fig.2: Sketch of the biofilm.

The pH is practically constant in the liquid-bulk and decreases in the biofilm moving towards the carried surface. The pH gradient is the result of the equilibrium between transport phenomena and microbiological conversions (Olivieri et al., 2011; Rai et al., 2012). Two regions may be identified: i) an external layer, 4.5<pH with cells under acidogenic phase; ii) and internal layer, pH<4.5 with cells under solventogenic phase. Acids are produced by cells present in the external layer, and they are reconverted by cells present in the inner layer. As a result, the acids net production depends on the relative depth of the two layers.

The acid production cells in the biofilm may be estimated taking into account the yields of butyric and acetic acids with respect to the biomass, and the rate of acids conversion. The yields may be assessed according to relationships reported by Napoli et al. (2012b):

$$Y_{X/L} = \frac{(3/46.62)Y_{ATP}}{1+(Y_{ATP}/46.62)}\frac{MW_X}{MW_L} \quad (6)$$

$$\mathrm{Y_{BA/L}} = \frac{1.5\mathrm{Y_{ATP}}}{1+\mathrm{Y_{ATP}}}\frac{\mathrm{MW_{BA}}}{\mathrm{MW_L}} \quad (7)$$

$$\mathrm{Y_{AA/L}} = \frac{\mathrm{Y_{ATP}}}{1+\mathrm{Y_{ATP}}}\frac{\mathrm{MW_{AA}}}{\mathrm{MW_L}} \quad (8)$$

where Y_{ATP} is the ATP fractional yield expressed as dry biomass for ATP molar unit. Working out eq.s (6), (7) and (8), it result:

$$Y_{Acids/X} = \frac{Y_{AA/L}+Y_{BA/L}}{Y_{X/L}} g_{Acids}/g_{DM} \quad (9).$$

The relationships among the yields and the specific growth rate reported by Napoli et al. (2012) were adopted to assess $Y_{Acids/X}$. The product among the dilution rate, the $Y_{Acids/X}$ and the biomass concentration under acidogenic phase ($X_{Acidogenic}$) yields the total acids production rate by acidogenic cells (W_{Acids}^{TOT}):

$$\mathrm{W_{Acids}^{TOT}} = \mathrm{D}\cdot\mathrm{X_{Acidogenic}}\cdot\mathrm{Y_{Acids/X}} \quad (10)$$

A fraction of produced acids is converted in solvents according to the reactions:

$$\mathrm{C_{12}H_{22}O_{11}\cdot H_2O + 2CH_3COOH \xrightarrow{+4ATP} 2CH_3COCH_3 + 2C_2H_5OH + 6CO_2 + 4H_2} \quad (11)$$

$$\mathrm{C_{12}H_{22}O_{11}\cdot H_2O + 2C_3H_7COOH \xrightarrow{+4ATP} 2CH_3COCH_3 + 2C_4H_9OH + 6CO_2 + 4H_2} \quad (12)$$

According to the stoichiometry of reactions (11) and (12), produced acetone is

equal to the sum of acetic and butyric acid converted:

$$[A] = [AA]^{UP} + [BA]^{UP} \quad (13)$$

where [A] is the acetone concentration, and $[j]^{UP}$ the concentration of the converted acid "j". Moreover, converted acids should fulfil the balance proposed by Desai et al. (1998):

$$\frac{[BA]^{UP}}{[AA]^{UP}} = 0.315\frac{[BA]}{[AA]} \quad (14)$$

where [j] is the concentration of the acid in the recator. Given the dilution rate at which the reactor is operated, the rate of the converted acids (W_{Acids}^{UP}) may be assed working out eq.s (13) and (14). The sum of the estimated rate of converted acids and of the measured rate of produced acids (W_{Acids}^{Net}) is W_{Acids}^{TOT}:

$$W_{Acids}^{TOT} = (W_{Acids}^{Net} + W_{Acids}^{UP}) \quad (15)$$

The concentration of acidogenic cells within the biofilm is estimated working out eq.s (10) and (15):

$$X_{Acidogenic} = \frac{W_{Acids}^{TOT}}{D \cdot Y_{Acids/X}} \quad (16)$$

RESULTS AND DISCUSSION

CWP pretreatment. Preliminary tests were carried out with solutions of raw CWP (40 g/L) without *C. acetobutylicum* inoculum (data not reported). The lactose was barely consumed and the conversion after 7 day was smaller than 2%. Although lactose uptake rate was inappreciable, results suggested that endogenous microorganisms were present and a light sterilization was planned.

The study regarding pretreatment of CWP (40 g/L) solutions was aimed at selecting the optimal conditions in terms of feedstock operability and butanol production. Denaturation of proteins with formation of aggregate/clots during thermal treatment of CWP should be avoided. Indeed, large clots may clog both pipes and the packed bed reactor during the continuous fermentation. The investigated pretreatment methods were:

- heat sterilization. CWP solution was sterilized in autoclave at 120 ° for 20 min;
- tyndalization. CWP solution was sterilized according to the following thermal program: 30 min at 80 °C; 24 hours at 37 °C; 30 min at 80 °C; 24 hours at 37 °C; 30 min 80 °C;
- dry tyndalization. CWP was sterilized according to following the thermal program: 60 min at 80 °C; 24 hours at 37 °C; 60 min at 80 °C; 24 hours at 37 °C; 60 min at 80 °C. The sterilized CWP was solubilized in sterilized dionized water.
- deproteinization. The CWP solution was deproteinized by heat treatment: 30 min at 90 °C. The precipitate was removed by centrifugation at 5'000 rpm, 30 min at 10 °C. The supernatant was sterilized in autoclave (120 °C, 20 min) to be used as fermentation medium.

Table 2 reports the results of the investigated pretreatment methods. The performance of each method was characterized in terms of clot formation and sterilization efficiency. The efficiency was expressed as the maximum concentration of butanol and residual concentration of acids measured in fermentation tests, compared with those measured in tests carried out without inoculum.

The analysis of table 2 shows that heat sterilization and tyndalization were characterized by good results in terms of sterilization efficiency.

Pretreatment Method		Max Butanol	Residual Acids	Clot Presence
Heat sterilization	40 g/L	6.4	2.7	YES
	control	0	0	
Tyndalization	40 g/L	6.3	2.7	YES
	control	0	0.1	
Dry Tyndalization	40 g/L	8.1	2.7	NO
	control	0	6.8	
Deproteinization	40 g/L	8.9	2.3	NO
	control	0	0	

Table 2: Cheese whey pretreatment methods. Main results.

However, both methods were discarded because a significant amount of clots formed. Both dry tyndalization and deproteneization methods were characterized by absence of clot formation and high butanol concentration. However, dry tyndalization was discarded because it was not successful in terms of sterilization.
Based on the results reported in table 2, deproteinization process was chosen as the best method to pretreat the CWP solution.

Biofilm reactor start-up. Tygon rings - 39.2 g – were adopted to prepare a packed bed reactor 8 cm high. The volume of the reactor was set at 100 mL by means of the overflow duct. The reactor was inoculated with actively growing culture. No chemicals was adopted to assist cell immobilization on the selected support. The start-up process was carried out adopting the synthetic medium with 25 g/L lactose.

Figure 3 reports time series of metabolite concentration and pH. The reactor was inoculated at t = 0, operated under batch conditions with respect to the liquid phase for 24 h (data non reported) and then under continuous conditions. The dilution rate was set at 0.40 h^{-1} and the pH was gradually increased from 5.0 to 5.5 to operate the fermentation under acidogenesis conditions (Napoli et al., 2011). A visible biofilm layer on carriers formed in about one week and at t= 7 day the dilution rate was increased (D = 0.8 h^{-1}) to promote biofilm production with respect to suspended cell growth. It is interesting to note that solvent production started even though the bulk pH was still 5.5. According to Fig. 2, the pH in the biofilm decreased moving from the liquid-biofilm towards the inner region (Qureshi et al., 2005; Napoli et al., 2010) promoting the solventogic shift of inner cells.

At t = 18.5 day the carriers appeared covered by abundant biofilm and a steady state conditions was approached. The total acid (acetic + butyric) yield was about 0.35 $g_{acid}/g_{lactose}$. The small difference of the measured total yield with respect to that assessed by Napoli et al. (2012b) may be due to the partial solvent production. The feeding stream was switched from lactose-synthetic medium to CWP solution (28 g/L lactose) to accustom cells to the complex medium. A new steady-state regime was approached in about three.

Altogether, the biofilm reactor start-up took about 21 days and a remarkable amount of active biofilm was formed.

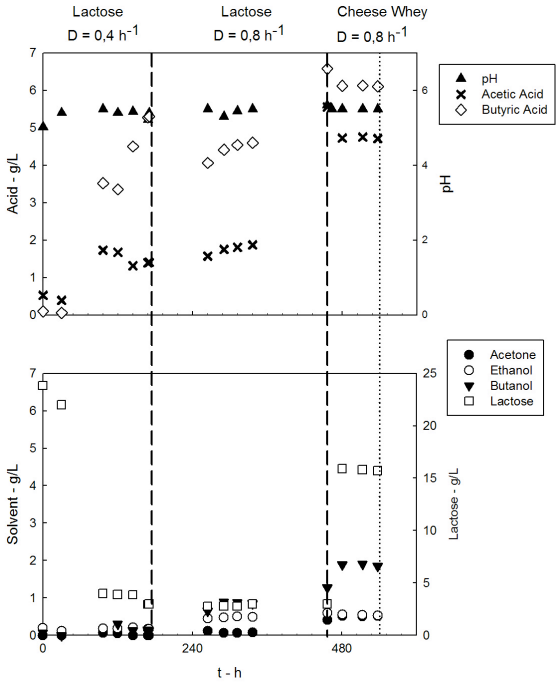

Fig.3 : Main data measured during PBR start-up. The vertical dotted line marks the instant at which the pH set-up of the controller was changed from 5.5 to 4.5.

Butanol production. The bioreactor operating conditions were changed at t = 21.7 day (Fig. 4) to produce butanol: pH=4.7, D=0.54 h^{-1}. The value of the pH was set according to previous investigations (Napoli et al., 2010). It was higher than the value at which cells shift to solvent production occurs, pH = 4 (Napoli et al., 2009), because pH and metabolites gradient across the biofilm (Fig. 2) is expected (Qureshi et al., 2005; Napoli et al., 2010).

The reactor approached a steady-state regime within 7 days. As expected, solvents and acids were continuously produced, confirming the co-existence of both *C. acetobutylicum* cells voted to produce ABE and cells committed to produce acids and biomass (Mollah and Stuckey, 1993; Napoli et al., 2009). PBR performances were characterized in terms of metabolite concentration, lactose conversion degree, yield of acids, butanol and ABE, productivity of butanol and of ABE, and butanol to solvents selectivity (Table 3). The performances were assessed working out the concentration of lactose and metabolites according the relationships reported in the "Procedure" section, provided that the steady-state was stabilized for at least 10 times the reactors mean residence time (1/D=1.9 h). The lactose concentration was 9 g/L, about half the affinity constant (28 g/L) assessed by Napoli et al. (2009).

Figure 4 reports main data measured during the continuous fermentation.

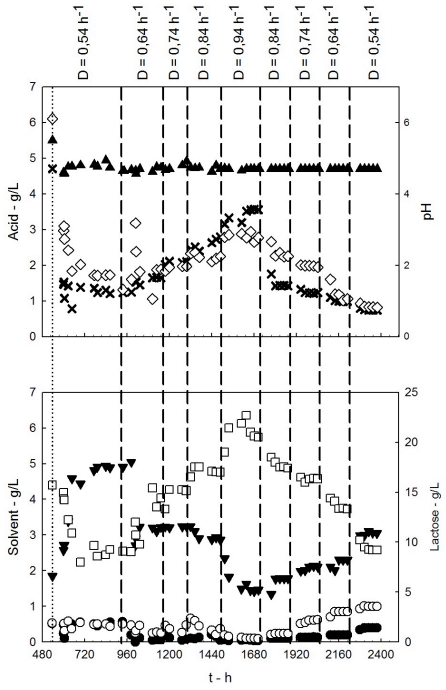

Fig.4: Data measured during the PBR operation as a function of the dilution rate. Feedstock: solution of CWP pretreated. Vertical dotted line: see caption of Fig. 4.

The dilution rate was steadily increased from 0.54 to 0.94 h^{-1} (Up phase). Table 3 and 4 report the PBR performances as a function of the dilution rate. The analysis of results reported in Figure 4 and Table 3 highlighted the issues reported hereinafter:

- butanol and solvent concentration, and lactose conversion degree (ξ_L) decreased with D. They were characterized by a maximum at D = 0.54 h^{-1}.
- except for the highest value of D investigated, butanol and ABE productivity as well as butanol and ABE yields were approximatevely constant with D. At D= 0.94 h^{-1} an abrupt reduction of solvent expression was assessed.
- butanol selectivity was quite high (0.92 g/g) and about constant with D. It is worthy to note that the selectivity was among the largest values reported in the literature;
- the concentration of acids and their yield and productivity increased progressively with D.

Results reported in Table 4 refer to steady-state cultures carried out at D decreasing between 0.94 h^{-1} and 0.54 h^{-1} (Down-phase). The analysis of the data in the Table confirms the effects of D highlighted with reference to the Up-phase investigation. However, PBR performances assessed during the Down-phase were not as high as those assessed during the Up-phase.

The biofilm PBR was stopped at the end of the Down-phase and the overall biomass concentration was 101.7 g_{DM} /L, that is a biomass to carrier ratio of 0.1 g_{DM}/g.

It is evident that the PBR performances decreased with the dilution rate under operating conditions investigated. The performances were almost constant for D varying between 0.64 and 0.84 h^{-1}, the PBR experienced its worst performances when D was set to 0.94 h^{-1}. Moreover, the acids productivity/concentration increased progressively with D and they were well pronounced at D=0.94 h^{-1}. From the butanol/solvent production point of view the best performances were assessed at the

lowest D investigated. Results are in agreement with those reported by Qureshi and Maddox (1987) even though performances assessed in the present investigation were better when PBRs are operated under close operating conditions (absence of yeast extract, 28 g/L lactose in the feeding).

The decrease of performances at high dilution rate deserves some comments.

From the kinetic point of view, the production rate of butanol/solvents should be favoured by high dilution rate. Indeed, the high lactose concentration coupled with the high feeding flow rate should increase of the production rate. From the biofilm structure point of view, the relationship between the PBR performances and D depends on the distribution of active cells present in the biofilm. High feeding flow rate is typically coupled with low lactose conversion, as reported in the present investigation and by Qureshi and Maddox (1987). Therefore, pH profile within the biofilm (Fig. 2) may be expected to be less steep and the layer thickness of acidogenic cells within the biofilm ($X_{Acidogenic}$) should increase: the boundary of the acidogenic layer should move towards the carrier surface. Indeed, Figure 5 supports this result: the $X_{Acidogenic}$ - assessed according to eq. (16) – increases with D.

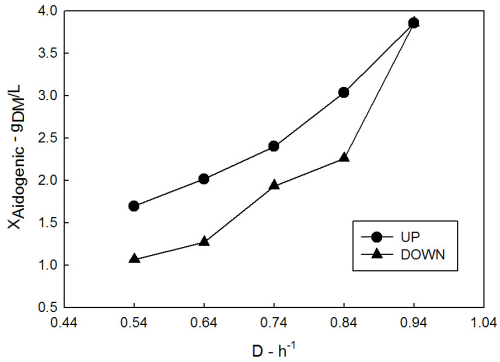

Fig.5: Estimated biomass concentration under acidogenic phase - eq. (16) - vs. the dilution rate.

Moreover, the gradual recover of solvent productivity as D decreased points out that biomass was still present in the PBR and ready to produce solvents when operating conditions were compatible with the conversion. It is worthy to note that the assessed acidogenic cells ranged between 1 and 4%$_W$ of the biomass present in the PBR. The biofilm complement consisted of clostridial-form cells, extracellular products, and spores.

The steady state at the D=0.94 h^{-1} was particularly interesting. Two issues may be listed as responsible of this behaviour.

The feeding flow rate – dilution rate 0.94 h^{-1} - was apparently too high and the stream may strip cells off the biofilm surface. Therefore, low butanol production rate and slightly high biomass in the effluent were observed at the outset of continuous operation at 0.94 h^{-1} dilution rate. However, high dilution rate also led to a high undigested level of lactose, as observed in the effluent (Fig. 4).

CONCLUSIONS

Butanol production by *Clostridium acetobutylicum* fermentation of cheese whey in a packed bed reactor (PBR) was investigated. Un-supplemented cheese whey was adopted. The cheese whey was just deproteinized to avoid clogging of the PBR.

The biofilm PBR was successfully operated for more than three months. The effects of the dilution rate (D) on the PBR performances was investigated. Solvents concentration, productivity rate and lactose conversion decreased with D. Under optimized conditions (D = 0.54 h^{-1}) performances were: butanol productivity 2.66 g/Lh, butanol concentration 4.93 g/L, butanol yield 0.26 g/g, butanol selectivity of the

overall solvents production 82%$_w$. The decrease of performances with D may be interpreted taking into account the fraction of acidogenic cells present in the biofilm.

NOMENCLATURE

L Lactose concentration
D Dilution rate
X biomass concentration
B butanol
ABE acetone-butanol-ethanol
AA acetic acid
BA butyric acid
ξ_L lactose conversion degree
$Y_{i/L}$ Lactose-to-"i-species" fractional yield coefficient.
W_i i-species productivity
Φ butanol selectivity

Operating Conditions					
D [h^{-1}]	0.54	0.64	0.74	0.84	0.94
pH	4.7				
Lactose in the feeding [g/L]	28				
Results					
Lactose [g/L]	9.1	14.4	15.2	17.0	20.6
ξ_L [%]	68	49	46	39	26
Acetone [g/L]	0.58	0.07	0.07	0.15	0.08
Butanol [g/L]	4.93	3.23	3.24	2.93	1.46
Ethanol [g/L]	0.50	0.25	0.47	0.21	0.24
Acetic acid [g/L]	1.23	1.65	2.10	2.73	3.57
Butyric acid [g/L]	1.32	1.89	1.97	2.19	2.65
Butanol productivity [g/Lh]	2.66	2.06	2.39	2.46	1.37
ABE productivity [g/Lh]	3.24	2.27	2.80	2.76	1.67
Butanol yield [g/g]	0.26	0.24	0.25	0.26	0.20
ABE yield [g/g]	0.32	0.26	0.29	0.30	0.22
Acids yield [g/g]	0.14	0.26	0.32	0.45	0.84
Butanol selectivity [g/g]	0.82	0.91	0.86	0.89	0.92

Table 3: Steady-state cultures of *C. acetobutylicum* (Up-phase).

Operating Conditions				
D [h^{-1}]	0.54	0.64	0.74	0.84
pH	4.7			
Lactose in the feeding [g/L]	28			
Results				
Lactose [g/L]	9.2	13.3	16.3	17.4
ξ_L [%]	67	53	42	38
Acetone [g/L]	0.4	0.2	0.13	0.1
Butanol [g/L]	3.06	2.3	2.13	1.79
Ethanol [g/L]	1.0	0.84	0.62	0.31
Acetic acid [g/L]	0.74	0.99	1.24	1.42
Butyric acid [g/L]	0.82	1.06	1.95	2.25
Butanol productivity [g/Lh]	1.65	1.47	1.58	1.5
ABE productivity [g/Lh]	2.41	2.14	2.14	1.85
Butanol yield [g/g]	0.16	0.16	0.18	0.17
ABE yield [g/g]	0.24	0.23	0.25	0.20
Acids yield [g/g]	0.08	0.14	0.27	0.35
Butanol selectivity [g/g]	0.67	0.70	0.72	0.85

Table 4: Steady-state cultures *of C. acetobutylicum* (Down-phase).

CONCLUSIONS

The study carried out during the present Ph.D. program aimed at investigating the Acetone-Butanol-Ethanol (ABE) production process by fermentation. The work has been carried out at the Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale of the Università degli Studi di Napoli 'Federico II'. The activities have been articulated along three paths: i) the characterization of the ABE fermentation process as regards kinetics and yields using different renewable resources (lignocellulosic biomass, high sugar content beverges and cheese whey); ii) the characterization of the ABE fermentation process according to the metabolic flux analaysis (MFA) and to the dynamic kinetic models; iii) the development of innovative continuous biofilm reactor for the ABE production.

A commercial clostridia strain has been investigated. *Clostridium acetobutylicum* DSM 792 has been selected for its ability to produce ABE with satisfactory selectivity towards the butanol.

The ABE fermentation process by adopting renewable resources. Characterization in terms of kinetics and yields.

The study was aimed at the assessment of both the kinetics and the yields of cell growth and metabolites produced during the fermentation of:

- sugars representative of hydrolized lignocellulosic biomass fermentation (glucose, mannose, arabinose and xylose);
- sugars representative of high sugar content beverages (glucose, fructose and sucrose);
- High sugar content beverages.

Batch cultures of free *C.acetobutyicum* cells have been investigated. Tests have been focused on the preliminary characterization of the fermentation with the aim of highlighting the relevant features of the process. The fermentation was characterized in terms of kinetics and yields under a wide interval of operating conditions (substrate concentration, nutrient concentrations, …).

Fermentation tests of *C. acetobutylicum* adopting sugars representative of hydrolized lignocellulosic biomass (glucose, mannose, arabinose, and xylose) have been succsessfull and they were aimed at investigating the optimal medium for butanol production. The activity has regarded the optimisation of the medium too: composition of the medium – species and concentration – has been changed in a wide interval of operating conditions. The analysis of the results has pointed out that the ABE production optimisation needs an accurate selection of the medium. The composition typically adopted for glucose is not optimal for all sugars. Productivity and butanol selectivity could be optimised by fine tuning of the medium composition taking into account reported results.

Batch fermentations of *Clostridium acetobutylicum* adopting high sugar content beverages (HSCBs: fruit juices, fruit syrups, soft drinks, sport drink) have been successful too. Main results were:

- *C. acetobutylicum* did not grow on raw HSCBs;
- *C. acetobutylicum* grew on HSCBs supplemented with yeast extract and mineral salts. The butanol maximum concentration was ≈10g/L. Glucose and fructose conversion was practically complete. Sucrose conversion was very low;

- tests carried out with supplemented hydrolysed HSCBs were characterized by solvents production and sugar conversion degree higher than those measured during just supplemented HSCBs.

Results pointed out that HSCBs are a promising feedstocks for the ABE fermentation process and that they may be converted almost completely. Considering the huge amount of HSCBs that should be disposed in each country, the investigated process has a twofold advantage: to reduce the disposal cost for these kind of beverages and to produce butanol that may be adopted as biofuel or as a chemical building block.

Characterization of the ABE fermentation process through MFA and dynamic kinetic models.

The MFA and the kinetic dynamic methodologies were adopted to characterize the ABE fermentation. Selected batch fermentation tests were carried out with the aim to charcaterize the time-evolution of the concentration of substrate, cells and metabolities. Tests were carried out at initial sugar concentration 60 g/L. The time-series of concentration were processed according the MFA and the kinetic model methodology. In particular:

- the MFA was adopted to investigate the role of the main reaction steps of the *C. acetobutylicum* metabolic pathway to convert reference sugars (glucose and xylose) of hydrolyzed lignocellulosic biomass into butanol. Results of batch fermentation tests carried out using glucose and xylose as carbon source were adopted for the flux assessment. The stoichiometric matrix of the model was characterized by a singularity that prevented the assessment of a unique set of fluxes of the primary metabolic activity. The non linear constrain proposed by Desai et al. (1999) relating the acetate and butyrate uptake fluxes was adopted to solve the model equation set. The MFA was proposed with reference to glucose and xylose as carbon source. The comparison of the assessed fluxes suggested the role of each reaction step as a function of the carbon source investigated. The analysis of the MFA has pointed out the roles played by acid formation enzymes in the complex primary metabolism of solventogenic clostridia. In particular, the effects of the carbon source on the relevance of each step of the acid formation/uptake pathway have been highlighted. The butyrate formation pathway plays a ket role both in the production of butyrate and in butyrate uptake without acetone formation.
- A kinetic dynamic model of acetone–butanol–ethanol (ABE) production by *Clostridium acetobutylicum* DSM 792 was proposed using the biochemical networks simulator COPASI. The effect of substrate was studied implementing the model with different sugars: glucose, mannose, fructose, sucrose, lactose, xylose and arabinose. If necessary, the metabolic pathway was modified according to the specific sugar. In particular, the Embden-Meyerhof-Parnas (EMP) pathway equations were used for hexose and disaccharide sugars while the pentose phosphate (PP) pathway equations were used for pentose sugars. The proposed model gave satisfactory results for each of the tested sugars: the squared correlation coefficient (r^2) between experimental time-course of metabolites and calculated ones ranged between 0.87 and 0.925

Development of innovative continuous biofilm reactor for the ABE production.

The study was aimed at the assessment of the butanol production in a *C. acetobutylicum* biofilm reactor. The activity aimed at the butanol production regarded

the design, set-up and operation of a biofilm fixed bed reactor. Unsupplemented cheese whey was adopted as renewable feedstock. Operating conditions of the continuous tests were selected to maximize the butanol production and butanol selectivity. In particular, main activities were:

- The selection of a pre-treatment process of the cheese-whey coupled with fermentation tests carried out under batch conditions to assess effects of the pre-treatment on fermentation performances;
- Tests with the pre-treated cheese whey to characterize the fermentation process in terms of butanol production and butanol selectivity;
- Design, set-up and optimization of a fixed bed biofilm reactor for cheese whey conversion;
- Tests with the biofilm reactor under continuous conditions.

The biofilm PBR was successfully operated for more than three months. The effects of the dilution rate (D) on the PBR performances was investigated. Solvents concentration, productivity rate and lactose conversion decreased with D. Under optimized conditions (D = 0.54 h^{-1}) performances were: butanol productivity 2.66 g/Lh, butanol concentration 4.93 g/L, butanol yield 0.26 g/g, butanol selectivity of the overall solvents production 82%$_w$. The decrease of performances with D may be interpreted taking into account the fraction of acidogenic cells present in the biofilm.

The success of operation of the Packed Bed Reactor (PBR) has fuelled the development of an innovative continuous biofilm reactor configuration: “carosello” of packed bed biofilm reactors connected in series. The reactor system was equipped with a device to switch the feeding at the reactor according to a pre-set sequence. A mathematical model to support the bioreactor system design was developed.

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. To assess the techno-economic feasibility of the process the complete knowledge of the kinetics and yields should be acquired (e.g. overall kinetics of solventogenesis phase, overall kinetics for a wide spectrum of sugars). Moreover, the recovery and concentration of butanol from the fermentation broth asks for an accurate investigation. Guidelines for the recovery and concentration of butanol may be deduced by process simulations. First attempts have been reported by (Liu and Fan, 2004; Napoli et al., 2012; Procentese et al., 2014). However, further study is required, experimental too.

REFERENCES

Afschar, A.S., Biebl H., Schaller K., Schugerl K. (1985). Production of acetone and butanolby *Clostridium acetobutylicum* in continuous culture with cell recycle. Appl. Microbiol. Biotechnol., 22:394-398.

Antoni, D., Zverlov, V., Schwarz, W. (2007). Biofuels from microbes. Appl. Microbiol. Biotechnol., 23-35.

Bahl, H., Gottwald, M., Kuhn, A., Rale, V., Andersch, W., Gottschalk, G. (1986). Nutritional factors affecting the ratio of solvents produced by *Clostridium acetobutylicum*, Appl. Environ. Microbiol., 52:169-172.

Bailey, J.E. (1991). Toward a science of metabolic engineering (Review). Science 252:1668-1675.

Bohlmann, G. (2007). Biobutanol. California: SRI Consulting.

Boynton, Z.L., Bennett, G.N., Rudolph, F.B., 1994. Intracellular concentrations of coenzyme A and its derivatives from *Clostridium acetobutylicum* ATCC 824 and their roles in enzyme regulation. Appl. Environ. Microbiol. 60, 39–44.

Brekke, K. (2007). Butanol: An Energy Alternative? Ethanol Today, pp. 36-39.

Cao, N.J., Xu, Q., Chen, C.S., Gong, C.S. (1994). Cellulose hydrolysis using zinc-chloride as a solvent and catalyst. Appl. Biochem. Biotechnol., 45/46:521-530.

Cascone, R. (2008) Biobutanol - A Replacement for Bioethanol? Chem. Eng. Prog., S4-S9.

Chen, C.K., Blaschek, H.P. (1999). Effect of acetate on molecular and physiological aspects of *Clostridium beijerinckii* NCIMB 8052 solvent production and strain degeneration, Appl. Environ. Microb. 65:499-505.

Chiao, J., Sun, Z. (2007). History of the acetone-butanol-ethanol fermentation industry in China: Development of continuous production technology. J. Molecular Microbiol. Biotechnol., 12-14.

Desai, R.P., Nielsen, L.K., Papoutsakis, E.T. (1998). Stoichiometric modeling of *Clostridium acetobutylicum* fermentations with non-linear constraints. J. Biotechnol., 71:191-205.

Desai, R.P., Harris, L.M.,Welker, N.E., Papoutsakis, E.T., (1999). Metabolic flux analysis elucidates the importance of the acid-formation pathways in reg- ulating solvent production by *Clostridium acetobutylicum*. Metab. Eng. 1, 206–213.

Doremus, M.G., Linden, J.C., Moreira, A.R. (1985). Agitation and pressure effect on acetone butanol fermentation, Biotechnol. Bioeng., 27:852-860.

Dow. (2006, May 2). Product Safety. Retrieved August 7, 2008, from DOW: http://www.dow.com/productsafety/finder/nbut.htm#ProdUses

DuPont. (2006, June 20). News Releases. Retrieved August 4, 2008, from DuPont: http://vocuspr.vocus.com/VocusPR30/Newsroom/Query.aspx?SiteName=DupontNew&Entity=PRAsset&SF_PRAsset_PRAssetID_EQ=102087&XSL=PressRelease&Cache=False

Dürre, P. (1998). New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. Appl. Microbiol. Biotechnol., 639-648.

Dürre, P. (2007). Biobutanol: An attractive biofuel. Biotechnol. J., 2:1525–1534.

Dürre, P. (2008). Fermentative Butanol Production. Annals of the New York Academy of Sciences, 353-362.

Dwidar, M., Lee, S., Mitchell, R.J. (2012). The production of biofuels from carbonated beverages. Appl. Energy 100:47–51.

Dyr, J., Protiva, J., Praus, R. (1958). Formation of neutral solvents in continuous fermentation by means of *Clostridium acetobutylicum*. p 210-226. In I. Malek (ed.), Continuous cultivation of microorganisms. A symposium. Cechoslovakian Academy of Sciences, Prague.

Ebener, J., Ezeji, T.C., Qureshi, N., Blaschek, H.P. (2003). Corn fiber hydrolysis and fermentation to butanol using *Clostridium beijerinckii* BA101, Proceedings of the 25th Symposium on Biotechnology for Fuels and Chemicals, Breckenridge, CO, USA.

Ennis, B.M., Maddox, I.S. (1985). Use of *Clostridium acetobutylicum* P262 for production of solvents from whey permeate, Biotechnol. Lett., 7:601-606.

Ezeji, T., Qureshi, N., Blaschek, H. (2004a). Butanol Fermentation Research: Upstream and Downstream Manipulations. The Chemical Record, 305-314.

Ezeji, T., Qureshi, N., Blaschek, H. (2004b). Acetone-butanol-ethanol production from concentrated substrate: reduction in substrate inhibition by fed-batch technique and product inhibition by gas stripping. Appl. Microbiol. Biotechnol., 63:653-659.

Ezeji, T., Qureshi, N., Blaschek, H. (2007a). Bioproduction of butanol from biomass: from genes to bioreactors. Biotechnol., 220-227.
Ezeji, T., Qureshi, N., Blaschek, H. (2007b). Butanol production from agricultural residues: impact of degradation products on *Clostridium beijerinckii* growth and butanol fermentation. Biotechnol. Bioeng., 97:1460-1469.
Ezeji, T.C., Qureschi, N., Blaschek H.P., (2007c). Production of acetone butanol (AB) from liquefied corn starch, a commercial substrate, using *Clostridium beijerinckii* coupled with product recovery by gas stripping, J. Ind. Microbiol. Biotechnol. 34, 771–777.
Ezeji, T., Blaschek, H. (2008). Fermentation of dried distillers' grains and solubles (DDGS) hydrolysates to solvents and value-added products by solventogenic clostridia. Bioresour. Technol., 99:5232– 5242.
Ezeji, T.C., Blaschek, H.P. (2010). Butanol production from lignocellulosic biomass, in: Biofuels from Agricultural Wastes and Byproducts, Wiley- Blackwell, Ames, Iowa, USA, pp. 19-37.
Ezeji, T.C., Richmond, C.B., Han, J. (2011). Stimulatory effects of calcium carbonate on butanol production by solventogenic *Clostridium* species, Continental J. Microbiol., 5:18-28.
Flickinger, M.C., Drew, S.W. (1999). Bioprocess technology: fermentation, biocatalysis, and bioseparation, Wiley-Blackwell, John Wiley & Sons Inc. vol 1–5.
Frick, C., Schügerl, K., 1986. Continuous acetone-butanol produc- tion with free and immobilized *Clostridium acetobutylicum*, Appl. Microbiol. Biotechnol. 25, 186–193.
Friedl, A. (2012). Lignocellulosic Biorefinery, Env. Eng. Manage. J., 11:75-79.
Gapes, J. (2000). The Economics of Acetone-Butanol Fermentation: Theoretical and Market Considerations. J. Mol. Microbiol. Biotechnol., 2:27-32.
Gheshlaghi, R., Scharer, J. M., Moo-Young, M., & Chou, C.P., 2009. Metabolic pathways of *clostridia* for producing butanol. Biotechnology advances, 27(6), 764–81.
Grube, M., Gapes, J. (2002). Application of quantative IR spectral analysis of bacterial cells to acetonebutanol-ethanol fermentation monitoring. Analytica Chimica Acta, 471:127-133.
Hongo, M. (1958). Change of solvent ratio by the addition of neutral red in the fermentation of pentose, Nippon Nogeikagaku Kaishi, 32 (1958) 219-223.
Hoops, S., Sahle, S., Gauges, R., Lee, C., Pahle, J., Simus, N., Singhal, M., Xu, L., Mendes, P., Kummer, U. (2006). COPASI — a COmplex PAthway SImulator. Bioinformatics 22, 3067-74.
Jacques, K., Lyons, T., Kelsall, D. (2003). The Alcohol Textbook 4th Edition. Nottingham: Nottingham University Press.
Jones, D.T., Woods, D.R. (1986). Acetone-butanol fermentation revisited, Microbiol. Rev., 50:484-524.
Junne, S., Gaida, S., Fischer, E., Götz, P. (2006). Metabolic responses of *Clostridium acetobutylicum* to changes in environmental conditions. Chemie Ingenieur Technik, 78:1405.
Junne, S. (2010). Stimulus response experiments for modelling product formation in *Clostridium acetobutylicum* fermentations, Dissertation TU Berlin.
Jurges, G., Survase, S., Berezina, O., Sklavounos, E., Linnekoski, J., Kurkijärvi, A., Väkevä, M., van Heiningen, A., Granström, T. (2012). Butanol production from lignocellulosics, Biotechnol. Lett., 34:1415-1434.
Kacser, H., Burns, J.A., 1973. The control of flux. In: Davies,D.D. (Ed.), Rate control of biological processes. Plenum,New York, pp. 429–432.
Kim, T.S., Kim, B.H. (1988). Electron flow shift in *Clostridium acetobutylicum* fermentation by electrochemically introduced reducing equivalent, Biotechnol. Lett., 10 :123–128.
Kolot, F.B. (1984). Immobilized cells for solvent production. Process. Biochem., 19:7-13.
Krouwel, P.G., Van der Laan, W.F.M., Kossen, N.W.F., (1980). Continuous production of n-butanol and isopropanol by immobilized, growing *Clostridium butylicum* cells. Biotechnol. Lett. 2, 253– 258.
Kumar, M., Gayen, K. (2011). Developments in biobutanol production: new insights, Appl. Energ., 88:1999-2012.
Lee, S.Y., Park, J.H., Jang, S.H., Nielsen, L.K., Kim, J., Jung, K.S. (2008a). Fermentative butanol production by *Clostridia*. Biotechnol. Bioeng., 101:209-228.
Lee, S., Cho, M., Park, C., (2008b). Continuous butanol production using suspended and immobilized *Clostridium beijerinckii* NCIMB 8052 with supplementary butyrate, Energy & Fuels. 22, 3459-3464.
Lee TM, Ishizaki A, Yoshino S, Furukawa K. (1995). Production of acetone, butanol, and ethanol from palm oil mill effluent using *C. saccharoperbutylacetonicum* N1-4. Biotechnol Lett, 17:649–54.
Leung, J.C.Y., Wang, D.I.C. (1981). Production of acetone and butanol by *Clostridium acetobutylicum* in continuous culture using free cells and immobilized cells. Proc. 2nd World Congr. Chem. Eng., 1:348-352.

Linden, J.C., Moreira, A.R., Lenz, T.G. (1986). Acetone and butanol, p. 915-931. In C.L. Cooney and A.E. Humphrey (ed.), Comprehensive biotechnology. The principles of biotechnology: engineering consideration. Pergamon Press, Oxford.

Liu, J., Fan, L.T., (2004). Downstream process synthesis for biochemical production of butanol, ethanol, and acetone from grains: generation of optimal and near-optimal flowsheets with conventional operating units, Biotechnology Progress. 20, 1518-1527.

Liu, Z., Ying, Y., Li, F., Ma, C., Xu, P. (2010). Butanol production by *Clostridium beijerinckii* ATCC 55025 from wheat bran, J. Ind. Microbiol. Biotechnol. 37:495-501.

Lopez-Contreras, A. (2003). Utilization of lignocellulosic substrates by solvent-producing Clostridia. Wageningen: Wageningen University.

Jones, D., Woods, D. (1986). Acetone-Butanol fermentation revisited. Microbiol. Rev., 484-524.

Maddox, I.S. (1989) The acetone butanol ethanol fermentation: recent progress intechnology. Biotechnol. Genetic Eng. Reviews, 7: 190-220.

Marchal, R., Rebeller, M., Vandecasteele, J.P. (1984). Direct bioconversion of alkali pretreated straw using simultaneous enzymatic hydrolysis and acetone butanol fermentation. Biotechnol. Lett., 6:523-528.

Mehaia, M.A., Cheryan, M. (1984). Ethanol production in a hollow fiber bioreactor using *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol., 20:100-104.

Meyer, C.L., Roos, J.W., Papoutsakis, E.T. (1986). Carbon monoxide gasing leads to alcohol production and butyrate uptake without acetone formation in continuous cultures of *Clostridium acetobutylicum*, Appl. Microbiol. Biotechnol. 24:159-167.

Mitchell, W. (1998). Physiology of carbohydrates to solvent conversion by *clostridia*. Adv. Microbiol. Physiol., 39:31-130.

Mollah, A.H., Stuckey, D.C., (1993). Maximizing the production of acetone–butanol in an alginate bead fluidized bed reactor using *Clostridium acetobutylicum*. J. Chem. Technol. Biotechnol. 56, 83.

Montoya, D., Spitia, S., Silva, E., Schwarz, W. (2001). Isolation of mesophilic solvent-producing clostridia from Colombian sources: physiological characterization, solvent production and polysaccharide hydrolysis. J. Biotechnol., 79:117-126.

Mussatto, I., Roberto, I. (2004). Alternatives for detoxification of diluted-acid lignocellulosic hydrolyzates for use in fermentative processes: a review. Bioresour. Technol., 93:1-10.

Mutschlechner, O., Swoboda, H., Gapes, J.R., (2000), Continuous two-stage ABE fermentationusing *Clostridium beijerinckii* NRRL B592 operating with a growth rate in the first stage vessel close to its maximal value, J. Mol.Microbiol. Biotechnol., 2:101-105.

Nakas, J.P., Schaedle, M., Parkinson, C.M., Coonley, C.E., Tanenbaum, S.W. (1983). System development for linked fermentation production of solvents from algal biomass. Appl. Environ. Microbiol., 46:1017-1023

Napoli, F. Development of an integrated process for butanol production, PhD thesis.

Napoli, F., Olivieri G., Russo, M.E., Marzocchella, A., Salatino, P. (2009). Bioenergy II: An Assessment of the Kinetics of Butanol Production by *Clostridium acetobutylicum*. Int. J. Chem. Reactor Eng., 7: A45.

Napoli, F., Olivieri G., Russo, M.E., Marzocchella, A., Salatino, P. (2010). Butanol production by *Clostridium acetobutylicum* in a continuous packed bed reactor. J. Ind. Microbiol. Biotechnol., 37:603-608.

Napoli, F., Olivieri G., Russo, M.E., Marzocchella, A., Salatino, P. (2011). Continuous lactose fermentation by *Clostridium acetobutylicum* - Assessment of acidogenesis kinetics. Bioresour. Technol., 102: 1608-1614.

Napoli, F., Olivieri, G., Russo, M.E., Marzocchella, A., Salatino, P. (2012a) Optimization of solvent recovery in the production of butanol by fermentation, Env. Eng. and Management J., 11:1499-1504.

Napoli, F., Olivieri, G., Russo, M.E., Marzocchella, A., Salatino, P. (2012b). Continuous lactose fermentation by *Clostridium acetobutylicum* —Assessment of energetics and product yields of the acidogenesis, Enz. Microb. Technol., 50:165-172.

Nimcevic, D., Gapes, J. (2000). The acetone-butanol fermentation in pilot plant and pre-industial scale. J. Mol. Microbiol. Biotechnol., 15-20.

Noguchi, T., Tashiro, Y., Yoshida, T., Zheng, J., Sakai, K., Sonomoto, K. Efficient butanol production without carbon catabolite repression from mixed sugars with *Clostridium saccharoperbutylacetonicum* N1-4, J. Biosci. Bioeng. in press.

Oceanethanol. (2007, February 21). Oceanethanol. Retrieved July 28, 2008, from Butanol Jet Fuel: http: //www.oceanethanol.com/CO2/Ocean_Ethanol/Entries/2007/2/21_Butanol_Jet_Fuel_From_CO2.html

Okamoto, M., Sakai, T., Hayashi, K., 1988. Biochemical switching device realizing McCulloch–Pitts type equation. Biol. Cybern. 58, 295–299.
Olivieri, G., Russo, M.E., Di Donato, A., Marzocchella, A., Salatino, P. (2010). Unstable steady state operations of substrate inhibited cultures by dissolved oxygen control. J. Biotechnol., 156:302–308.
Olivieri, G., Russo, M.E., Marzocchella, A., Salatino, P., (2011). Modeling of an Aerobic Biofilm Reactor with Double-Limiting Substrate Kinetics: Bifurcational and Dynamical Analysis Biotechnol. Prog. 27:1599-1613.
Papoutsakis, E.T. (1984). Equations and calculations for fermentations of butyric acid bacteria. Biotechnol. Bioeng. 26:174-187.
Peguin, S., Delorme, P., Goma, G., Soucaille, P. (1994). Enhanced alcohol yields in batch cultures of *Clostridium acetobutylicum* using a three-electrode potentiometric system with methyl viologen as electron carrier, Biotechnol. Lett., 16 :269-274.
Pierrot, P., Fick, N., Engasser, J.M. (1986) Continuous acetone-butanol fermentation with high productivity by cell ultrafiltration and recycling. Biotechnol. Lett., 8:253-256.
Procentese, T. Guida, F. Raganati, G. Olivieri, P. Salatino, A. Marzocchella. Process Simulation of Biobutanol Production from Lignocellulosic Feedstocks. Submitted for publication in Chemical Engineering Transactions.
Qureshi, N., Maddox I.S., (1987). Continuous solvent production from whey permeate using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. Enzyme Microbial Technol., 9:668–671.
Qureshi, N., Paterson, A.H.J., Maddox, I.S., (1988). Model for continuous production of solvents from whey permeate in a packed bed reactor using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. Appl. Microbiol. Biotechnol., 29:323– 328.
Qureshi, N., Blaschek, H. P. (1999). Butanol recovery from model solution / fermentation broth by pervaporation : evaluation of membrane performance. Biomass and Bioenergy, 17:175–184.
Qureshi, N., Blaschek, H.P. (2000). Economics of butanol fermentation using hyper-butanol producing *Clostridium beijerinckii* BA101, Trans. I. Chem. E., 78(Part C):139-144.
Qureshi, N., Schripsema, J., Lienhardt, J., Blaschek, H.P., (2000). Continuous solvent production by *Clostridium beijerinckii* BA101 immobilized by adsorption onto brick. World J. Microb. Biot., 16:337-382.
Qureshi, N., Blaschek H.P. (2001). Evaluation of recent advances in butanol fermentation,upstream, and downstream processing, Bioproc. Biosys. Eng., 24:219-226.
Qureshi N, Meagher MM, Huang J, Hutkins RW., (2001). Acetone butanol ethanol (ABE) recovery by pervaporation using silicalite-silicone composite membrane from fed-batch reactor of *Clostridium acetobutylicum*.J Memb Sci.187:93–102.
Qureshi, N., Maddox I.S., (1987). Continuous solvent production from whey permeate using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. Enz. Microbial. Technol., 9:668-671.
Qureshi, N., Maddox, I.S. (1988). Reactor design for the ABE fermentation using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. Bioprocess. Eng., 3:69-72.
Qureshi, N., Schripsema, J., Lienhardt, J., Blaschek, H.P. (2000). Continuous solvent production by *Clostridium beijerinckii* BA101 immobilized by adsorption onto brick, World J. Microbiol. Biotechnol., 16:377-382.
Qureshi, N., Annous, B.A., Ezeji, T.C., Karcher, P., Maddox, I.S., (2005). Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates. Microbial Cell Factories. 4:24.
Raganati, F., Curth, S., Gotz, P., Olivieri, G., Marzocchella, A., (2012). Butanol production from lignocellulosic-based hexoses and pentoses by fermentation of Clostridium acetobutylicum. Chemical Engineering Transaction 27: 91-96.
Raganati, F., Olivieri, G., Procentese, A., Russo, M.E., Marzocchella, A., Salatino, P. (2013). Butanol production by bioconversion of cheese whey in a continuous packed bed reactor, Bioresour. Technol., 138:259-265.
Raganati, F., Procentese, A, Olivieri, G., Russo, M.E., Salatino, P., Marzocchella, A. (2013). Biobutanol Production from Hexose and Pentose Sugars, submitted for pubblication.
Rai, V., Upadhyay, R.K., Thakur, N.K.. (2012). Complex Population Dynamics in Heterogeneous Environments: Effects of Random and Directed Animal Movements, Int J Nonlinear Sci. Numer. Simul., 13:299–309.
Ramey, D., Yang, Z. (2004). Production of Butyric Acid and Butanol from Biomass. Ohio: Environmental Energy Inc.

Saravanan, V., Waijers, D.A., Ziari, M., Noordermeer, M.A. (2010). Recovery of 1-butanol from aqueous solutions using zeolite ZSM-5 with a high Si/Al ratio; suitability of a column process for industrial applications, Biochem. Eng. J., 49:33–39.

Scott, A., Bryner, M. (2006). Alternative Fuels Rolling Out Next Generation Technologies. Chemical Week, pp. 17-21.

Shaheen, R., Shirley, M., Jones, D. (2000). Comparative fermentation studies of industrial strains belonging to four species of solvent-producing clostridia. J. Mol. Microbiol. Biotechnol., 2(1):115-124.

Schuster, K.C., Goodacre, R., Gapes, J.R., Young, M. (2001) Degeneration of solventogenic *Clostridium* strains monitored by Fourier transform infrared spectroscopy of bacterial cells. J. Ind. Microbiol. Biotechnol., 27:314–321.

Servinsky, M.D., Kiel, J.,T., Dupuy, N., F., Sund, C., J., (2010). Transcriptional analysis of differential carbohydrate utilization by *Clostridium acetobutylicum*. Microbiol 156, 3478-3491.

Shinto H, Tashiro Y, Yamashita M, Kobayashi G, Sekiguchi T, Hanai T, et al. (2007). Kinetic modeling and sensitivity analysis of acetone–butanol–ethanol production. J Biotechnol, 131:45–56.

Shinto H, Tashiro Y, Yamashita M, Kobayashi G, Sekiguchi T, Hanai T, et al. (2008). Kinetic study of substrate dependency for higher butanol production in acetone–butanol–ethanol fermentation. Process Biochemistry, 43(12):1452-1461.

Smith, J., Workman, J. (2007). Alcohol for motor fuels. Retrieved 07 29, 2008, from Colorado State University: http://www.ext.colostate.edu/PUBS/FARMMGT/05010.html

Stephanopoulos, G., Sinskey, A. (1993). Metabolic engineering methodologies and future prospects. Trends Biotechnol., 11:392-396.

Syed, Q. (1994). Biochemical Studies on Anaerobic Fermentation of Molasses by *Clostridium acetobutylicum*. Lahore: University of the Punjab.

Tashiro, Y., Takeda, K., Kobayashi, G., Sonomoto, K., Ishizaki, A., Yoshino, S. (2004). High butanol production by *Clostridium saccharoperbutylacetonicum* N1-4 in fed batch culture with pH-Stat continuous butyric acid and glucose feeding method, J. Biosc. Bioeng., 98:263-268.

Toerien, D.F., Hattingh, W.H.J., (1969). Anaerobic digestion I. The microbiology of anaerobic digestion. Water Res. 3(6):385-416.

Varga, E., Klinke, H.B., Reczey, K., Thomsen, A.B. (2004). High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol. Biotechnol. Bioeng., 88:567–574.

Villadsen, J. (2007) Innovative technology to meet the demands of the white biotechnology revolution of chemical production, Chem. Eng. Sci., 62:6957-6968.

Voget, C.E., Mignone, C.F., Ertola, R.J. (1985). Butanol production from apple pomace. Biotechnol. Lett., 7:43-46.

Voget, C.E., Mignone, C.F., Ertola, R.J. (1989). Influence of temperature on solvents production from whey. Biotechnol. Lett., 7:607-610.

Votruba, J., Volesky, B., Yerushalmi, L., (1986), Mathematical model of a batch acetone-butanol fermentation. Biotechnol. Bioeng. 28: 247-255.

Walton, M., & Martin, J. (1979). Production of butanol-acetone by fermentation. Microbial Technol., 1:187-209.

Welsh, F. W., I. A. Veliky. (1984). Production of acetone butanol from acid whey, Biotechnol. Lett., 6:61-64.

Welsh, F.W., Veliky, I.A. (1986). The metabolism of lactose by *Clostridium acetobutylicum*. Microbial Technol., 8:43-46.

Welsh, F.W., Williams, R.E., Veliky, I.A., (1987). Solid carriers for a *Clostridium acetobutylicum* that produces acetone and butanol. Enzyme Microb. Technol., 9:500-502.

Wiley, A.S., Johnson, M.J., Mc Coy, E., Peterson, W.H. (1941). The fermentation of waste sulfite liquor. Ind. Eng. Chem., 35:606-610.

Zeng, A.P., Ross, A., Biebl, H., Tag, C., Gunzel, B., Deckwer, W.D., (1994). Multiple product inhibition and growth modeling of *Clostridium butyricum* and *Klebsiella pneumoniae* in glycerol fermentation. Biotechnol. Bioeng. 44, 902–911.

Zverlov, V., Berezina, O., Velikodvorskaya, G., Schwarz, W. (2006). Bacterial acetone and butanol production by industrial fermentation in the Soviet Union: Use of hydrolyzed agricultural wastefor biorefinery. Appl Microbiol Biotechnol, 587-597.

Articles

F. Raganati, S. Curth, P. Götz, G. Olivieri, A. Marzocchella (2012). Butanol production from lignocellulosic-based hexoses and pentoses by fermentation of *Clostridium acetobutylicum*. Chemical Engineering Transactions, 27:91-96**.**

F. Raganati, G. Olivieri, A. Procentese, M.E. Russo, P. Salatino, A. Marzocchella (2013). Butanol Production By Bioconversion Of Cheese Whey In A Continuous Packed Bed Reactor. Bioresource Technology, 138: 250-265.

F. Raganati, G. Olivieri, ME. Russo, A. Marzocchella, P. Salatino. Butanol Production by *Clostridium acetobutylicum* in a Continuous Packed Bed Reactor Fed with Cheese Whey. Chemical Engineering Transactions, 32, p. 937-942**.**

F. Raganati, A. Procentese, F. Montagnaro, G. Olivieri, M.E. Russo, P. Salatino, A. Marzocchella. Butanol Production From High Sugar Content Beverages, Submitted for publication.

F. Raganati, P. Götz, G. Olivieri, A. Marzocchella. P. Salatino. Butanol production from hexoses and pentoses by fermentation of *Clostridium acetobutylicum*. Submitted for publication.

F. Raganati, A. Procentese, G. Olivieri, M.E. Russo, P. Götz, A. Marzocchella, Analysis Of Substrate Effect On Butanol Production By *Clostridium acetobutylicum* With A Kinetic Dynamic Model. Submitted for pubblication.

F. Raganati, A. Procentese, G. Olivieri, M.E. Russo, P. Salatino, A. Marzocchella. (2014). MFA Of *Clostridium Acetobutylicum* Pathway: The Role Of Glucose And Xylose On The Acid Formation/Uptake. Accepted for publication in Chemical Engineering Transactions vol. 38.

F. Raganati, A. Procentese, G. Olivieri, P. Salatino, A. Marzocchella. (2014). Biobutanol Production from Hexose and Pentose Sugars. Accepted for publication in Chemical Engineering Transactions vol. 38.

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. Submitted for pubblication in Bioresource Technology.

A. Procentese, T. Guida, **F. Raganati**, G. Olivieri, P. Salatino, A. Marzocchella. (2014). Process Simulation of Biobutanol Production from Lignocellulosic Feedstocks. Accepted for publication in Chemical Engineering Transactions vol. 38.

Conference presentations

F. Raganati, G. Olivieri, P. Götz, A. Marzocchella, P. Salatino. Biobutanol production from pentose-hexose sugar mixtures. BIO World Congress on Industrial Biotechnology. June 16-19, 2013 Montréal, Canada.

F. Raganati, G. Olivieri, ME. Russo, A. Marzocchella, P. Salatino. Butanol Production by *Clostridium acetobutylicum* in a Continuous Packed Bed Reactor Fed with Cheese Whey. 11th Int. Conf. Chemical & Process Engineering. 2-5 June, 2013, Milan (IT).

F. Raganati, G. Olivieri, ME. Russo, A. Marzocchella, P. Salatino. Butanol production by fermentation process. Convegno Nazionale GRICU. Montesilvano (PE) 16-19 Settembre 2012.

F. Raganati, P. Götz, G. Olivieri, A. Marzocchella. *Clostridium acetobutylicum* Fermentation on Lignocellulosic-based Hexoses and Pentoses for Butanol Production. Int. Conference on the Genetics, Physiology and Biotechnology of Solvent- and Acid-forming Clostridia 10-12th September 2012. Nottingham, UK

F. Raganati, S. Curth, P. Götz, G. Olivieri, A. Marzocchella. Butanol production from lignocellulosic-based hexoses and pentoses by fermentation of *Clostridium acetobutylicum*. IBIC 2012 24-27 June, 2012, Palermo (IT)**.**

F. Raganati, G. Olivieri, ME. Russo, A. Marzocchella, P. Salatino. Biobutanol Production From High Sugar Content Wastewaters. BioEenergy IV: Innovations in Biomass Conversion for Heat, Power, Fuels, and Chemicals 9-14 June, 2013, Otranto (IT)

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. Accepted for partecipation to IBIC 2014.

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

A. Procentese, T. Guida, **F. Raganati**, G. Olivieri, P. Salatino, A. Marzocchella. Process Simulation of Biobutanol Production from Lignocellulosic Feedstocks. Accepted for partecipation to 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).

P. Götz, K. Karstens, H. Niebelschütz, R. Görlitz, G. Olivieri, **F. Raganati**, A. Marzocchella. (2014) Continuous production of biobutanol: Design and evaluation of bioreactor configurations. Accepted for 3rd BioProScale Symposium "Inhomogeneities in large-scale bioprocesses: System biology and process dynamics", Berlin 2-4 April.

Permanence at research structures in Europe

21 September – 21 October 2012. Research group leaded by Prof. Peter Götz. Department of Life Sciences and Technology / Bioprocess Engineering, Beuth University of Applied Sciences Berlin,

16 September – 19 September 2012. Workshop on "Bacterial secretion" CNRS, Marsiglia.

Awards

"Waste to Energy" 2013 Altran Foundation Award. Project "Waste to Biofuels: production of biobutanol from waste(water) streams of food/beverage industries"

Attended course/seminaries

1 **Nico Martens**: Lecture 1 "Immunotherapy: an overview"; Lecture 2 "Beyond Antibodies: Design of New Recombinant Molecules for Therapy". April 2011.
2 **Dan Gavrilescu**: "Potential of biomass and recovered paper as raw materials for bioethanol". May 2011.
3 **Mariana Gavrilescu**: "Biosorption and bioaccumulation in environmental remediation". May 2011.
4 **Gennaro Marino**: "Advanced Proteomics". May 2011.
5 **Thierry Tron**: "Bio and bioinspired catalysts". June 2011.
6 **Renata Piccoli**: "Ordine e disordine nelle proteine". Novenbre 2011.
7 **Antonio Marzocchella**: "Stima economica di una linea di processo biotecnologica". December 2011.
8 **Scuola di Dottorato GRICU**. Mathematical Methods for Chemical Engineering/Nanotechnologies September 2011.
9 **"Bacterial Secretion" Workshop.** September 2012.
10 **G. Marino, F. Pane, F. Pennacchio, R. Rao, G. Sannia: "**Advanced biotechnology". May-June 2013.
11 **R. Piccoli, F. Rossi, P. Giardina: "**Ordine e disordine nelle proteine". January 2012.
12 **A. Marzocchella:** Biotecnologie per l'energia. December 2012
13 **V. Calabrò:** RNA non coding e genomica funzionale. October 2012.
14 **Lars Rehmann:** Biochemical Conversion of Biomass for the Production of Value Added Chemicals. June 2013.
15 **Peter Götz:** Dynamic Processes in Microbial Metabolism. May 2013.
16 **Dr Rajeev K Sukumaran and Dr Binod Parameswaran:** Training course: "Second Generation Bioethanol". October 2013.