
***DEVELOPMENT OF AN INTEGRATED
PROCESS FOR BUTANOL
PRODUCTION***

Fabio Napoli

Dottorato in Scienze Biotechnologiche – XXII ciclo
Indirizzo Biotechnologie Industriali
Università di Napoli Federico II



Dottorato in Scienze Biotecnologiche – XXII ciclo
Indirizzo Biotecnologie Industriali
Università di Napoli Federico II



***DEVELOPMENT OF AN INTEGRATED
PROCESS FOR BUTANOL
PRODUCTION***

Fabio Napoli

Dottorando: Ing. Fabio Napoli

Relatore: Prof. Antonio Marzocchella
Prof. Piero Salatino

Coordinatore: Prof. Ettore Benedetti

INDEX

ABSTRACT	1
LIST OF RELATED PUBLICATIONS	2
RIASSUNTO DEL LAVORO DI TESI	3
1. INTRODUCTION.....	8
1.1 A.B.E. History	8
1.2 State of the Art.....	9
1.3 Metabolic Pathway.....	16
1.4 Stoichiometry	20
1.5 The New Butanol Enterprises	22
1.6 Biofilm reactor.....	23
1.6.2 Biofilm reactors	25
1.7 Scope of the Thesis	27
2 THEORETICAL FRAMEWORK.....	29
2.1 Continuous fermentation with microfiltration unit to confine cells.....	32
3. BATCH CULTURES.....	34
3.1 An Assessment of the Kinetics of Butanol Production by <i>Clostridium acetobutylicum</i>	34
4 CONTINUOUS CULTURES	47
4.1 Butanol Production by <i>Clostridium acetobutylicum</i> – I. Acidogenesis Kinetics	47
4.2 Butanol Production by <i>Clostridium acetobutylicum</i> – II. Mass and Energy Yields under Acidogenesis Phase	62
4.3 Solvotogenic Phase: Preliminary Tests and Data Analysis.....	76
5 BIOFILM REACTOR	80
5.1 Butanol Poduction in a Continuous Packed Bed Reactor by <i>Clostridium acetobutylicum</i>	80
6. ECONOMIC EVALUATION	92
6.1 Optimization of Solvent Recovery in the Production of Butanol by Fermentation	92
7. CONCLUSIONS	99
NOMENCLATURE	100
REFERENCES.....	101

ABSTRACT

The research program addressed the conversion of lactose to Acetone-Butanol-Ethanol by *Clostridium acetobutylicum* DSMZ 792. The research pathway is ultimately targeted at the development of: a) novel and effective tools for the characterization of the peculiar conversion process, cells growth under acidogenesis conditions and solvents production under solventogenesis conditions; b) biofilm reactors for the intensified ABE production. The study was articulated in three paths: i) the characterization of the ABE fermentation process as regards kinetics and yields; ii) the development of a continuous biofilm reactor for the ABE production; iii) the economic assessment of the process for the recover and concentration of solvents.

Characterization of the ABE fermentation process as regards kinetics and yields.

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 with 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 and the solventogenesis phases. 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. Those with the second 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.

Development of a continuous biofilm reactor for the ABE production

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 has been design, 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 largest value reported in the literature regarding the lactose conversion: 4.43 g/Lh. The butanol selectivity with respect solvents (88%_w) was among the largest value reported in the literature.

Economic assessment of the process for the recover and concentration of solvents.

The study was aimed at investigating the economical feasibility of butanol recovery from ABE fermentation by means of an unconventional flowsheet.

The activities were focused on the selection of unit operations, their simulation by means of the Aspen Plus® process simulator. The flowsheet was optimized assuming the venture profit as objective function.

LIST OF RELATED PUBLICATIONS

- Napoli F.**, G. Olivieri, A. Marzocchella, P. Salatino
Bioenergy II: An Assessment of the Kinetics of Butanol Production by *Clostridium acetobutylicum*
International Journal of Chemical Reactor Engineering, 2009, vol. 7, A45
- Napoli F.**, G. Olivieri, M.E. Russo, A. Marzocchella, P. Salatino.
Butanol Production by *Clostridium acetobutylicum* - I. Acidogenesis Kinetics
Biotechnology and Bioengineering, *submitted*
- Napoli F.**, G. Olivieri, M.E. Russo, A. Marzocchella, P. Salatino
Butanol Production by *Clostridium acetobutylicum* – II. Mass and Energy Yields under Acidogenesis Phase
Biotechnology and Bioengineering, *submitted*
- Napoli F.**, A. Marzocchella, P. Salatino
Optimization of Solvent Recovery in the Production of Butanol by Fermentation
Biochemical Engineering Journal, *submitted*
- Napoli F.**, G. Olivieri, A. Marzocchella, M.E. Russo, P. Salatino
Butanol Production in a Continuous Biofilm Reactor by *Clostridium acetobutylicum*
Progress Biochemistry, *submitted*

Proceedings

- Napoli, F.**, Olivieri G., Marzocchella A., Salatino P. (2008) Assessment of Kinetics for Butanol Production by *Clostridium acetobutylicum*. In: 1st Int. Conf. on Industrial Biotechnology (G. Marino, E. Bardone and A. Viglia, Eds), 273-280, AIDIC, Italy
- Napoli F.**, Marzocchella A., Salatino P. (2008) Optimization of solvent recovery in the production of butanol by fermentation, Graduate School of Chemical Engineering Gricu 2008 Le Castelle (KR) Italy
- Napoli F.**, G. Olivieri, M.E. Russo, A. Marzocchella, P. Salatino (2009) "Production of Biobutanol by *Clostridium Acetobutylicum*", "Combustion Colloquia 2009 - 32nd Meeting on Combustion", curato da R. Ragucci, Napoli (IT) April 26-28, 2009, ISBN 978-88-88104-10-2, paper n. IV-2
- Napoli F.**, Olivieri G., Marzocchella A., Russo M.E., Salatino P., (2010) Assessment of Kinetics for Butanol Production by *Clostridium Acetobutylicum*, "2st Int. Conf. on Industrial Biotechnology" curato da E. Bardone e A. Viglia AIDIC (Italy) Padova April 2010. *submitted*

Communications to International Conferences

- Napoli, F.**, Olivieri G., Russo M.E., Marzocchella A., Salatino P. (2009) *Butanol Production by Clostridium Acetobutylicum: Assessment of Acidogenesis Kinetics*, 8th WCCE, Montreal, (Canada)
- Napoli, F.**, Marzocchella A., Salatino P. (2009) *Optimization of Solvent Recovery in Production of Butanol*, 8th WCCE, Montreal, (Canada)
- Napoli, F.**, Marzocchella A., Salatino P. (2009) *Butanol Production assessment by Clostridium acetobutylicum using continuous reactor equipped with microfiltration unit*. Bioenergy - II: Fuels and Chemicals from Renewable Resources. March 8-13, 2009 - Rio de Janeiro (Brazil)

RIASSUNTO DEL LAVORO DI TESI

Introduzione. L'attuale scenario energetico-ambientale ha determinato un rinnovato interesse per la produzione di Acetone-Butanolo-Etanol (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 delle tecnologie 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 acclerate potenzialità dei sistemi microbici nel produrre sostanze di interesse industriale, lo sviluppo di processi è limitato da due aspetti: i) la carenza di informazioni riguardanti le equazioni costitutive del processo (crescita microbica, produzione di solventi, fattori di resa, selettività); ii) la 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; Brew, 2006).

Scopo della tesi. Lo studio svolto durante il corso di DdR ha riguardato lo sviluppo di un processo per la produzione di butanolo mediante *Clostridium acetobutylicum* DSM 792 per la valorizzazione energetica di reflui dell'industria agro-alimentare. Il ceppo commerciale selezionato permette di studiare il processo produttivo, di caratterizzarlo, individuarne stadi limitanti e soluzioni reattoristiche ottimali. Le principali direttrici dell'attività di ricerca condotta sono riportate di seguito.

A) Caratterizzazione cinetica del processo di produzione di butanolo. L'attività ha riguardato la caratterizzazione sia in termini cinetici che in termini di resa della crescita del microrganismo e della produzione di butanolo e di altri metaboliti di interesse. Lo studio si è avvalso sia di reattori classici (continui e discontinui) ad agitazione meccanica e/o pneumatica, sia di reattori continui a perfetta miscelazione (CSTR) di concezione innovativa equipaggiati con unità di microfiltrazione per il contenimento delle cellule. Le campagne di prove condotte sono state finalizzate al reperimento di dati idonei a formulare un'espressione cinetica globale e ad acquisire informazioni per la formulazione di un modello cinetico dettagliato.

B) Immobilizzazione dei batteri su supporti granulari. L'attività ha previsto: i) la selezione di supporti granulari idonei per il processo investigato; ii) la messa a punto di un protocollo di immobilizzazione.

C) Selezione e sviluppo di una configurazione reattoristica. Particolare attenzione è stata posta alla possibilità di utilizzare reattori a biofilm eserciti in continuo in condizioni controllate.

D) Analisi economica del processo di produzione del butanolo. L'attività ha riguardato la stima economica di massima del processo di produzione di ABE per via fermentativa

avvalendosi delle metodologie classiche dell'Ingegneria Chimica e di software di *flowsheeting* di processo.

Le campagne sperimentali sono state condotte adottando come mezzo di coltura soluzioni di estratto di lieviti (YE=5 g/L) e lattosio a concentrazione variabile tra 2 e 100 g/L. Alcune prove sono state condotte con siero di latte.

Caratterizzazione cinetica del processo di produzione di butanolo.

Prove condotte in discontinuo. La crescita del microrganismo e la produzione di solventi nel corso di prove condotte in *batch* sono state caratterizzate in termini di pH, concentrazione di metaboliti (acidi e alcoli), carbonio totale organico disciolto e concentrazione di microrganismi. Sono altresì state condotte delle osservazioni al microscopio per verificare l'omogeneità delle specie microbiche presenti. Le misure sono state elaborate al fine di stimare, al variare delle condizioni operative, la velocità di crescita del microrganismo, la velocità di produzione dei metaboliti, la velocità di consumo del substrato e le rese del processo.

L'analisi delle misure risolte nel tempo della concentrazione cellulare e di metaboliti in colture *batch* ha permesso di identificare e caratterizzare due fasi ben distinte del processo: la fase di acidogenesi e la fase di solventogenesi.

La fase di acidogenesi è caratterizzata dalla crescita esponenziale del microrganismo associata alla produzione di acido acetico e acido butirrico con diminuzione progressiva del valore di pH. L'accumulo di acidi e la conseguente diminuzione del pH, fino al valore soglia di 4.0, giocano un ruolo chiave nella transizione alla fase di solventogenesi. Tale fase è caratterizzata dall'arresto della crescita cellulare e dalla produzione di solventi sia per conversione diretta del substrato sia per conversione degli acidi precedentemente prodotti.

Acidogenesi. Al variare della concentrazione iniziale di lattosio nell'intervallo investigato (2-100 g/L) risulta che: i) la velocità specifica di crescita del microrganismo così come il rapporto acido butirrico e acido acetico sono costanti e uguali a, rispettivamente, a 0.29 h^{-1} e 1.5 su base molare; ii) l'ammontare di lattosio consumato durante la fase di acidogenesi è costante e corrispondente ad un abbattimento della concentrazione nella misura di 4 g/L, quando disponibile; iii) i fattori di resa in biomassa ed acidi riferiti alla sola fase di acidogenesi sono costanti. In particolare alla fine della fase di acidogenesi, e purché la concentrazione iniziale di lattosio sia superiore a 4 g/L, si riscontra sempre biomassa e acidi totali a concentrazione di $0.7 \text{ g}_{\text{DM}}/\text{L}$ e 1.5 g/L , rispettivamente.

L'invarianza della velocità di crescita con la concentrazione di substrato permette di ipotizzare una cinetica di crescita microbica di ordine zero rispetto alla concentrazione di substrato.

Solventogenesi. La concentrazione finale di butanolo aumenta con la concentrazione di lattosio presente all'inizio della fase di solventogenesi. La resa in butanolo è caratterizzata da un massimo di $0.2 \text{ g}_B/\text{g}_L$ a concentrazione iniziale di lattosio di 50 g/L. La velocità di produzione specifica di butanolo in funzione della concentrazione di lattosio (L) è ben descritta da una cinetica alla Monod:

$$r_B^* = 0.13 \frac{L}{28 + L}, \text{ g}_B/\text{hg}_{\text{DM}} \quad (1)$$

dove r_B^* è la massima velocità specifica di produzione di butanolo calcolata all'insorgere della fase di solventogenesi.

I risultati riguardanti il processo fermentativo nel suo complesso (acidogenesi/solventogenesi) mostrano che il grado di conversione di lattosio e la selettività in butanolo decrescono con la concentrazione iniziale di lattosio.

Prove *batch* condotte con siero caseario hanno mostrato risultati simili a quelli ottenuti nel corso delle prove *batch* condotte con il mezzo sintetico.

Prove condotte in continuo. La cinetica di crescita di *C. acetobutylicum* durante la fase di acidogenesi è stata investigata in un reattore a perfetta miscelazione esercito in continuo e in condizioni controllate di pH. I risultati hanno mostrato che il processo è caratterizzato da inibizione da prodotti (ac. acetico, AA – ac. butirrico, BA – acetone, Ac – etanolo, Et – butanolo, B) e che esiste un valore soglia di concentrazione per ciascun metabolita (AA_{max} , BA_{max} , Ac_{max} , Et_{max} , B_{max}) oltre la quale non vi è crescita microbica. In accordo con il modello proposto da Loung (1985), la cinetica di crescita per *C. acetobutylicum* è espressa dalla:

$$\mu = \mu_{max} \frac{L}{K_L + L} \left(1 - \frac{AA}{AA_{max}}\right)^{n_{AA}} \left(1 - \frac{BA}{BA_{max}}\right)^{n_{BA}} \left(1 - \frac{Ac}{Ac_{max}}\right)^{n_{Ac}} \left(1 - \frac{Et}{Et_{max}}\right)^{n_{Et}} \left(1 - \frac{B}{B_{max}}\right)^{n_B} \quad (2)$$

dove μ e μ_{max} sono la velocità specifica di crescita ed il suo valore massimo, rispettivamente, K_L la costante di saturazione del termine alla Monod.

Prove condotte per verificare l'effetto del pH sulle cinetiche del processo hanno mostrato che la concentrazione idrogenionica non influenza la capacità inibitoria dei metaboliti ma solo la massima velocità di crescita in accordo alla relazione:

$$\mu_{max} = \frac{\bar{\mu}_{max}}{1 + \frac{H^+}{K_H} + \frac{K_{OH}}{H^+}} \left(1 - \frac{10^{(pH_{OPT} - pH)}}{10^{\Delta pH}}\right) \quad (3)$$

dove $\bar{\mu}_{max}$, K_H e K_{OH} , pH_{OPT} e ΔpH sono parametri.

La determinazione della concentrazione massima dei metaboliti e degli altri parametri che compaiono nelle equazioni (2) e (3) è stata condotta adottando un'opportuna campagna di prove con l'intento di minimizzarne il numero complessivo delle stesse. In particolare: i) la prima stima di μ_{max} e K_L è stata basata sui dati caratteristici di prove condotte al limite della condizione di *wash-out* per minimizzare l'effetto inibitorio dei metaboliti; ii) la prima stima della concentrazione massima dei metaboliti è stata basata sui dati caratteristici di prove condotte con terreni addizionati di singoli metaboliti a concentrazione progressivamente crescente.

I 14 parametri del modello cinetico (2)-(3) sono stati definitivamente determinati mediante algoritmi di inferenza parametrica (a corredo del software EXCEL) e risultano:

$\bar{\mu}_{max}$ (h ⁻¹)	K_H 10 ⁻⁴ (M)	K_{OH} 10 ⁻⁷ (M)	pH_{OPT}	ΔpH	K_L (g/L)	AA_{max} (mg/L)	BA_{max} (mg/L)	B_{max} (mg/L)	Ac_{max} (mg/L)	Et_{max} (mg/L)	n_i	n_B
1.06	4.33	5.04	4.9	1.5	1.34	1557	2995	16500	38500	69000	1	0.47

Il basso valore di K_L è in accordo con i risultati ottenuti nel corso delle prove *batch* che indicano la sostanziale costanza della velocità specifica dalla concentrazione di lattosio per $L > 4$ g/L. E' inoltre interessante notare come la velocità massima specifica di crescita microbica registrata nelle prove in continuo sia oltre tre volte la velocità specifica di crescita riscontrata in modalità *batch*.

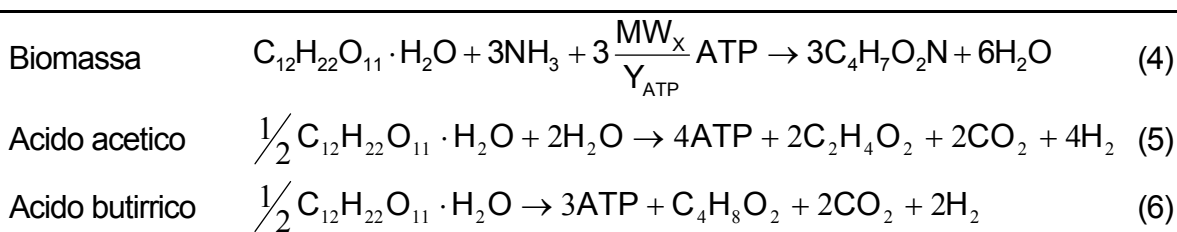
La cinetica di produzione di butanolo mediante *C. acetobutylicum* durante la fase di solventogenesi è stata investigata in un reattore a perfetta miscelazione, equipaggiato con un'unità di microfiltrazione, esercito in continuo e in condizioni controllate di pH. La massima velocità di produzione del butanolo è risultata 0.4 g_B/Lh - velocità di produzione specifica di 75 mg_B/g_{DM}h – a concentrazione di lattosio di circa 5 g/L. In generale la velocità specifica di

produzione di butanolo stimata nel corso delle prove in continuo è risultata 2-3 volte maggiore rispetto quelle rilevate in *batch* in condizioni operative simili.

Fattori di resa in prodotti e in ATP

I risultati delle prove condotte in continuo sono stati elaborati per acquisire informazioni riguardanti un modello cinetico dettagliato, comprensivo delle rese in biomassa e in prodotti al variare della velocità di crescita.

La fase di acidogenesi può essere ricondotta a tre reazioni:



dove $C_4H_7O_2N$ rappresenta la composizione elementare della biomassa (peso molecolare 101 g/mole) ed Y_{ATP} il fattore di resa in ATP, definito come rapporto tra la massa batterica prodotta per mole di ATP consumata.

Le prove condotte sia in condizioni *batch* che in continuo hanno evidenziato che il rapporto molare tra acido butirrico e acido acetico è 1.5 nelle condizioni di esercizio indagate. Alla luce di questa osservazione la combinazione della (5) e della (6) fornisce l'unica reazione:



La quantità di ATP consumata nel corso della produzione della biomassa (4) è prodotta durante la formazione degli acidi conformemente alla reazione (7).

Dall'analisi delle (4) e (7) risulta che per poter stimare i fattori di resa in biomassa ed in acidi è necessario conoscere il fattore di resa in ATP al variare delle condizioni di esercizio. Dalla (7) risulta:

$$Y_{ATP} = 29.5 \times Y_{X/A} \quad (8)$$

dove 29.5 è la massa in grammi di acidi prodotti per mole di ATP prodotta ed $Y_{X/A}$ il rapporto tra le velocità di produzione della biomassa e degli acidi.

I dati di fattore di resa in ATP e di velocità specifica di crescita sono stati elaborati in conformità al modello proposto da Pirt (1982):

$$Y_{ATP} = Y_{ATP}^{MAX} \frac{\mu}{\mu + Y_{ATP}^{MAX} \times m_1 \left(1 - \frac{\mu}{\mu_{max}} \right)} \quad (9)$$

dove Y_{ATP}^{MAX} è la massima resa di ATP, m_1 le moli di ATP necessarie per il mantenimento di un grammo di biomassa nell'unità di tempo. Nelle condizioni di esercizio indagate risulta:

$$Y_{ATP}^{MAX} = 29.1 \text{ g}_{DM}/\text{mole}_{ATP} \text{ e } m_1 = 0.012 \text{ mole}_{ATP}/\text{h g}_{DM}.$$

Immobilizzazione e reattore a biofilm continuo

Sono state condotte prove preliminari riguardanti la capacità delle cellule di *C. acetobutylicum* di formare biofilm su supporti granulari costituiti da materiali di varia natura: vetro, Tygon®, pomici e gel di silice. Il Tygon® grazie alle sue proprietà idrofobiche si è rivelato un ottimo supporto per le cellule di *C. acetobutylicum*.

Sono state allestite diverse tipologie di reattori bifasici eserciti in continuo in condizioni di portata (velocità di diluizione, D) e pH controllato. Le migliori prestazioni sono state registrate con un reattore a letto fisso. Le prove sono state condotte a D variabile tra 0.54 h^{-1} e 2.4 h^{-1} (2.5 volte la massima velocità specifica di crescita), pH tra 4 a 6 e concentrazione di lattosio variabile tra 5 e 10 g/L.

Il reattore a biofilm stabilizzato su granuli di Tygon® è stato esercito in continuo con successo per oltre un mese. Nel corso della prova la massima produttività di butanolo e la massima selettività in butanolo sono state $4.5\text{ g}_B/\text{Lh}$ e 88%, rispettivamente, misurate a $D=0.97\text{ h}^{-1}$ e $\text{pH}=5.0$. Considerando che alla fine della prova la biomassa immobilizzata è risultata pari a $0.16\text{ g}_{\text{DM}}/\text{g}_{\text{supporto}}$ e assumendo che tale valore non sia molto diverso da quello stabilizzato nelle condizioni ottimali, la massima produttività specifica è risultata $54\text{ mg}_B/\text{g}_{\text{DMh}}$.

Recupero del butanolo: linea di processo e analisi economica

Il recupero e concentrazione del butanolo - ed in generale dei solventi - dal brodo di fermentazione è reso particolarmente difficile e dispendioso per le sue basse concentrazioni e per la sua bassa tensione di vapore rispetto all'acqua.

Nell'ambito dell'attività svolta è stato formulato un possibile schema di processo per il recupero e la concentrazione del butanolo. Lo schema proposto, alternativo agli schemi tradizionali, è stato oggetto di analisi tecnico-economica preliminare. La linea di processo è costituita da operazioni unitarie finalizzate a concentrare il butanolo, che viene successivamente separato dagli altri componenti liquidi, nativi o aggiunti, per distillazione. Quantificati di processo relativi allo schema proposto sono stati ottenuti con l'ausilio del software di *flowsheeting* Aspen Plus®. La valutazione di convenienza economica della linea di recupero del butanolo è stata effettuata assumendo il *venture profit* come funzione-obiettivo. L'analisi dei risultati mostra che impianti di produzione di ABE per via fermentativa sono economicamente vantaggiosi per capacità di trattamento superiori a 250 t/giorno di siero caseario.

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 operations on the environment; ii) to produce bulk-chemicals by eco-sustainable processes; iii) to diversify the fuels source. The development of processes using biocatalyst systems – microorganisms or enzymes produced by them – is based on the conversion potential with respect to 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 necessary for processes operated at high throughput. Therefore, the development of industrial scale bio-based processes asks for innovative technologies aimed at intensified operations.

1.1 A.B.E. History

The Acetone-Butanol-Ethanol (ABE) fermentation has very far root in the time, crossing the main events of last century. The success of this productive process and its development has been strongly affected by events like World Wars, Israel birth and the development of petrochemical industry.

The firsts investigation on the butanol production by anaerobic bacteria may be dated back to the end of the 19th century (Ross, 1961). The shortage of natural rubber stimulated the interest in producing synthetic rubber. In 1910 the Strange and Graham Ltd. in England started a project to produce synthetic rubber - butadiene or isoprene - with the cooperation of Prof. Weizmann adopting butanol or isoamyl alcohol as chemical brick. In 1912 Weizmann suggested that the production of butanol or isoamyl alcohol by fermentation was a central issue for the success of the production process of the synthetic rubber. He isolated and studied a number of cultures, one of which named BY and later *Clostridium acetobutylicum*. This microorganism had a number of unique properties including the ability to use a variety of starchy substances and to produce high yields of butanol and acetone with respect to other cultures known at that time.

The industrial development of the ABE fermentation was changed radically by the outbreak of the First World War in August 1914. The increased demand of acetone as the colloidal solvent for nitrocellulose - used to manufacture cordite for the munitions – directed the efforts towards this solvent. In 1916 Weizmann started the first tests on a pilot-scale plant to produce acetone by fermentation at the Lister Institute of London. The promising results persuaded the Royal Naval Cordite Factory to built-up an industrial plant at Poole in Dorset and to revamp six distilleries in Great Britain for products recovery and concentration. In short time the starchy feedstocks in Europe started running out and the fermentation plant was moved to Canada. The plant was in operation until armistice in 1918 and produced 3'000 tons of acetone and 6'000 tons of butanol.

After the war Weizmann addressed all the gratitude of the Allies for his activity in pursuing his wish: a home for the Jews in Palestine. After the Balfour Declaration, Weizmann became the leader of the Zionist Organization and the first President of

Israel.

The acetone-butanol fermentation plant built-up by Weizmann was taken over by a group of American businessmen that founded the Commercial Solvents Corporation of Maryland. The plant was run with success even though the production rate was drastically reduced as a consequence of a bacteriophage infection. In December 1923, a new plant was built-up to fulfill the increasing demand for butanol - in spite of phage infection - and consisted of 32 fermenters for a total volume of 189 m³. Nevertheless, a further expansion of plants was necessary between 1924 and 1927. Altogether, 148 fermenters were operating for an overall productivity of 100 tons of solvents per day (Ross, 1961).

During this pioneer phase the activity also regarded the screening of strains. Particular attention was paid to strains able to ferment molasses at high concentration of sugars. The strain named CSC no. 8 was able to ferment in media containing 6% sugar and was largely adopted in the fermentation plants. Particularly interesting was a strain able to ferment 6.5% sugars media and to produce up to 2%_w solvents contributing to the reduction of the production cost. In fact, production processes based on this strain were characterized by the use of cheaper substrates and less complex distillation process to recover and concentrate the solvents (Flickinger and Drew, 1999). The several fermentation companies that bloomed since the 1930s started to create wide collections of strains able of carrying out competitive fermentations. Between 1935 and 1941 at least 18 patents were issued covering different strains.

The II World War re-fuelled the demand for acetone devoted to the munitions manufactures. The large part of the fermentation plants dedicated to produce solvents was converted for the ABE process. Moreover, to fulfill the increasing demand of the ABE solvents particular attention was paid to the improvements of the ABE fermentation section and to the ABE recovery and concentration. In particular, semi-continuous fermentations were successfully tested and multiple-column continuous distillation units were adopted for the solvent recovery.

After the Second World War, the interest in the ABE fermentation was characterized by a continuous decline for a twofold issue. First, the petrochemical industry grew at an unprecedented rate and by the late 1950s competition between the fermentation and chemical process had become very acute. Second, the molasses began to be used in substantial amounts in cattle feed, particularly in the United States. Competitive market resulted in the escalation of the price of molasses, affecting drastically the economical balance of the AB fermentation processes (Flickinger and Drew, 1999).

The petroleum crisis in 70's and its successive recurrence has renewed the interest in butanol fermentation. Today the social-economic scenarios together with recent developments in downstream processing and biotechnology techniques as well as the advances in fermentation systems have contributed to give a new impulse to research in butanol production.

1.2 State of the Art

Among the saccharolytic butyric acid-producing *clostridia*, there are a number of species capable of producing significant amounts of neutral solvents during the later stage of a batch fermentation under appropriate operating conditions (*C. saccharoperbutylacetonicum*, *C. acetobutylicum*, *C. beijerinckii*, *C. aurantibutyricum*). Solventogenic *clostridia* are anaerobic strains able to metabolize a great variety of substrates, pentose, hexoses, mono-, di-, and polysaccharides (Flickinger and Drew,

1999). Typically, the strains selected in industrial fermentation depend on the nature of the raw material available, the required ratio among the solvents, the need for additional nutrients, and phage resistance (Ross, 1961). *C. acetobutylicum* has been successfully adopted for the production of acetone and butanol.

Even though the ABE fermentation processes are known since the end of the 19th century (see § 1.1) their diffusion at industrial scale is still affected by several limits that compromised the economic competition with respect to petrochemical industry. Main issues regarding the development of the ABE fermentation processes at industrial scale are hereafter reported.

Feedstocks. The use of conventional feedstocks as corn and molasses make the fermentation uncompetitive with the petrochemical processes (Lenz and Moreira., 1980). Typically, the cost of the substrates is about 60% of the overall production cost of the ABE (Ross, 1961). On other hand, the use of feedstocks as corn, maize and molasses is directly in competition with the food market. Therefore, the increase of the raw material price - pumped by the high request of butanol like fuel - will have socio-political consequence too (Gapes, 2000).

Solvent concentration. Butanol and the other metabolites are highly toxic for the biological systems (Jones and Woods, 1986; Xiaoping and Tsao, 1994). As a consequence, batch processes are characterized by low solvent volumetric productivity because the active biomass accumulation and the maximum solvents concentrations in the fermenter (about 2%) are very low. Moreover, the recovery of solvents by distillation is extremely expensive because of the large amount of energy required.

Yield and selectivity in butanol. The yield of the bioconversion process in the butanol is typical low (theoretical value about 0.41 g/g). The selectivity in butanol with respect to the solvents typically ranges between 0.6 and 0.8 g/g. Moreover, the process suffers of the inevitable presence of unconverted substrates that affects the economical balance with respect to the feedstocks, and the downstream operation to recover and concentrate the solvents (Xiaoping and Tsao, 1994; Meyer and Papoutsakis, 1989; Mutschlechner *et al.*, 2000).

Feedstocks

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. The high carbohydrates content of some wastewater streams, e.g. the agro-industrial effluents, asks for expansive 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.* (7) adopted apple pomace as feedstock in a fermentation process to produce butanol production. 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*. Researchers (Nakas *et al.*, 1983) pointed out that the microalgae was successfully converted with a production of about 16 g/L of solvent, provided a 4% glycerol 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) pointed out that the solvent production was not inhibited by the salt present in algal concentrates. However, he 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. Cellulosic biomass contains about 20 to 40% hemicellulose (Flickinger and Drew, 1999), with D-xylose being the major constituent of hemicellulose. *C. acetobutylicum* is capable of utilizing all of the prevalent sugars present in wood hemicellulose and cellulose hydrolysates (Ezeji *et al.*, 2007).

Hemicellulose is a plant cell wall polysaccharide that is localized mostly in cell wall middle lamella. It is in close association with cellulose, lignin, and pectic materials. The methods utilized during the process pretreatment strongly depend on the structure of the raw material utilized.

Pretreatment of lignocellulosic materials prior to utilization is a necessary step in the biomass-to-butanol conversion processes. The objective of the pretreatment is to render biomass components more accessible to either chemical or 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 of 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 treated than hardwood materials. Typically, softwood biomasses are recalcitrant because 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. In general, the pretreatment processes must be efficient, cost effective, and environmental friendly. Without enter in the details of the pretreatment unit operations, the main features of lignocellulosic pretreatments are reported in the following.

The large part of the pretreatment technologies economically possible comprises a combination of mechanical size reduction, alkali swelling, acid hydrolysis, steam and other explosion techniques to disrupt the fibers, e.g. exposure to supercritical fluids. The processes comprise 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 typically very slow and compromise the process throughput.

The ideal technology for the lignocellulosic-biomass pretreatment comprises the dissolution of the solid materials into aqueous substrate. Many cellulose solvents at high concentrations can penetrate into the cellulose crystalline structure to dissolve 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, and very expensive to recover. Therefore, 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 and 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.

Corn fiber is a renewable resource available in significant quantities 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). 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 to simple sugars, using economic technologies. Dilute sulfuric-acid pretreatment can be applied to agricultural residues to bring about 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.) (Zaldivar *et al.*; 1999; Varga *et al.*, 2004).

Sulfite waste liquors from the pulp and paper industries contain glucose, xylose, and arabinose and have been investigated as alternate substrates for AB 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 AB 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%_{wt}.

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 developing of alternative methods for dispose cheese-whey. The presence of lactose at a remarkably concentration makes possible the use of whey as substrates for chemical bulk production by fermentation. The substrates would be available at very low price, or the fermentation process might be considered as a disposal technology and a rate should be cashed for unit of mass fermented.

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). At this low concentration the solution is not appropriate as substrate for a large spectrum of microorganisms without a preliminary concentration. However, the lactose concentration is suitable for the AB fermentation since product toxicity limits the amount of sugar converted.

The use of whey and lactose as substrates in AB fermentation has been investigated by a number of 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; yield 0.23-0.41 g_{solvents}/g_{substrate}). On the other hand, the concentration of total solvents reached at the end of the cheese-whey fermentations is lower than that reached at 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.

Reactor configuration

Design and operation of bio-reactors play an important role in the biochemical industry. The knowledge of the rate of reactions, transport phenomena, hydrodynamics, and operating conditions allows to improve the 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 growth 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, at the beginning of the butanol production on large scale batch fermentation was commonly used because it was: i) typically adopted in fermentation processes; ii) the better process 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 m³. Of course, the AB fermentation process can not be economically competitive on these bases.

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

Continuous cultures can be utilized with the same efficiency as batch cultures as regards both solvents concentration and yield and are often characterized by improved productivity. The main disadvantage of this reactor typology 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. At dilution rate of 0.1 h^{-1} , they obtained a production of $15.9\text{ g}_{\text{solvents}}/\text{L}$, 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 $2.2\text{ }10^{-2}\text{ h}^{-1}$. The performance of the reactor system was: solvent concentrations of $\sim 15\text{ g/L}$ (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 (Nimevic and Gapes, 2000).

Even though 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 AB fermentation requires inevitably the achieving of better levels of process intensification in 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 AB bio-production. With reference to microorganism-based processes, intensification is usually achieved:

- by the containment of the biocatalyst within the reactor, by means of semipermeable membranes;
- by 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 either 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 value added and therefore any clever device for an economical intensification is welcome.

The production of solvents during the non-growing solventogenic phase in batch culture suggests that immobilized cell systems may be more suited for solvent production than continuous culture utilizing free cells. The advantages of immobilized cell systems include: i) the physical retention of the cells in the matrix, 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 just at the desired rate. 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.

Product recovery

The limitation imposed by the high cost of solvent recovery by distillation has stimulated research into alternative methods of solvent recovery. Processes reported in the open literature regard: selective adsorption or absorption, vacuum fermentation, membrane separation, extraction by means of aqueous two-phase systems.

The extractive fermentation is an interesting process based on the continuous removal of products from the broth fermentation. The main advantage of this

technology is the possibility to stabilize solvent concentrations in the fermenter sufficiently low that do not inhibit the bioconversion. The extractive fermentation systems investigated may be classified into two groups: a) the first group considers the *in situ* extraction; b) the second, the extraction of solvents in an apparatus external with respect to the fermenter, having the medium working as a shuttle between the fermenter and the extractor (Linden *et al.*, 1986). Several studies are available on the nature of the extractive liquid. The polyoxyalkylene ethers appear very effective in the extraction. They are characterized by: i) distribution coefficients ranging between 1.5 and 3; ii) absence of toxicity towards microorganisms (Griffith *et al.*, 1983). Taya *et al.* (1985) reported that oleyl alcohol (cis-9-octadecen-1-ol) was an excellent extracting solvent for butanol.

The adsorption of solvent on solids *in situ* is an interesting process for solvent recovery and at the same time to improve fermentation performance. In fact, the solvent adsorbed on the solid phase is removed from the liquid phase, reducing the solvent concentration in the bulk and then the process inhibition degree in the broth. Activated carbon and silicalite - a zeolite-like solid - have been investigated to adsorb solvents in the fermenter and to release them by thermal desorption. Qureshi *et al.*, (2005) reported that 252 mg of butanol per g of active carbon could be adsorbed from AB fermentation liquors.

Aqueous two-phase extractive systems provide an alternative to organic/aqueous extraction (Rajni, 2001). Jarzbski *et al.* (1992) have investigated acetone and butanol production by *C. acetobutylicum* in aqueous two-phase systems obtained by supplementing dextran and polyethylene glycol to the fermentation broth.

Pervaporation appears to be a process characterized by strong potentiality. The liquid is confined by a solid membrane and diffuses through it. Then, the diffused liquid evaporates at the external surface of the membrane in an inert gas stream of by applying a vacuum. The solvents are recovered by condensation on a chilled surface. The economic feasibility of large-scale processes is still depending on availability of the membrane technology. In particular main limits are: membrane cost and membrane biofouling.

The listed solvent recover technologies offer the spectrum of the possible unit operations that may be assorted in the process dedicated to the solvent recover and concentration. The success of the possible combination depends on the characteristics of the broth and on peculiarity energetic resources available at the plant site. Liu and Fan (2004) and Oudshoorn *et al.* (2009) have reported interesting strategies to discriminate among possible recover process layouts. However, no attempt regarding economical optimization is reported in the literature.

1.3 Metabolic Pathway

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 (acidogenic phase). As the acids and hydrogen ion concentrations increase (pH decrease), the metabolism of cells shifts to solvent production (solventogenic phase) and acidogenesis cells - able to reproduce itself - shift to solventogenesis state with a morphologically change. In particular, active cells become endospores unable to reproduce themselves and able to metabolize acids and substrate to produce solvents.

Therefore, two different pathways must be taken into account for *clostridia* cells during solvent production: one for acidogenesis phase and one for solventogenesis phase.

Acidogenesis phase

Figure 1.1 reports the metabolic pathways of lactose by *C. acetobutylicum* under acidogenesis phase. The lactose flows in the cells by diffusion then it is split by β -Galactosidase in two monomers: glucose and galactose. The glucose is oxidized to pyruvate through the well known Embden–Meyerhof–Parnas (EMP) pathway. The galactose is still converted in pyruvate but through a longer pathway: i) the *galactokinase* convert the galactose in galactose 1-phosphate by consuming ATP, 1 mole of ATP for mole of galactose; ii) glucose 1-phosphate is produced by *uridyl*

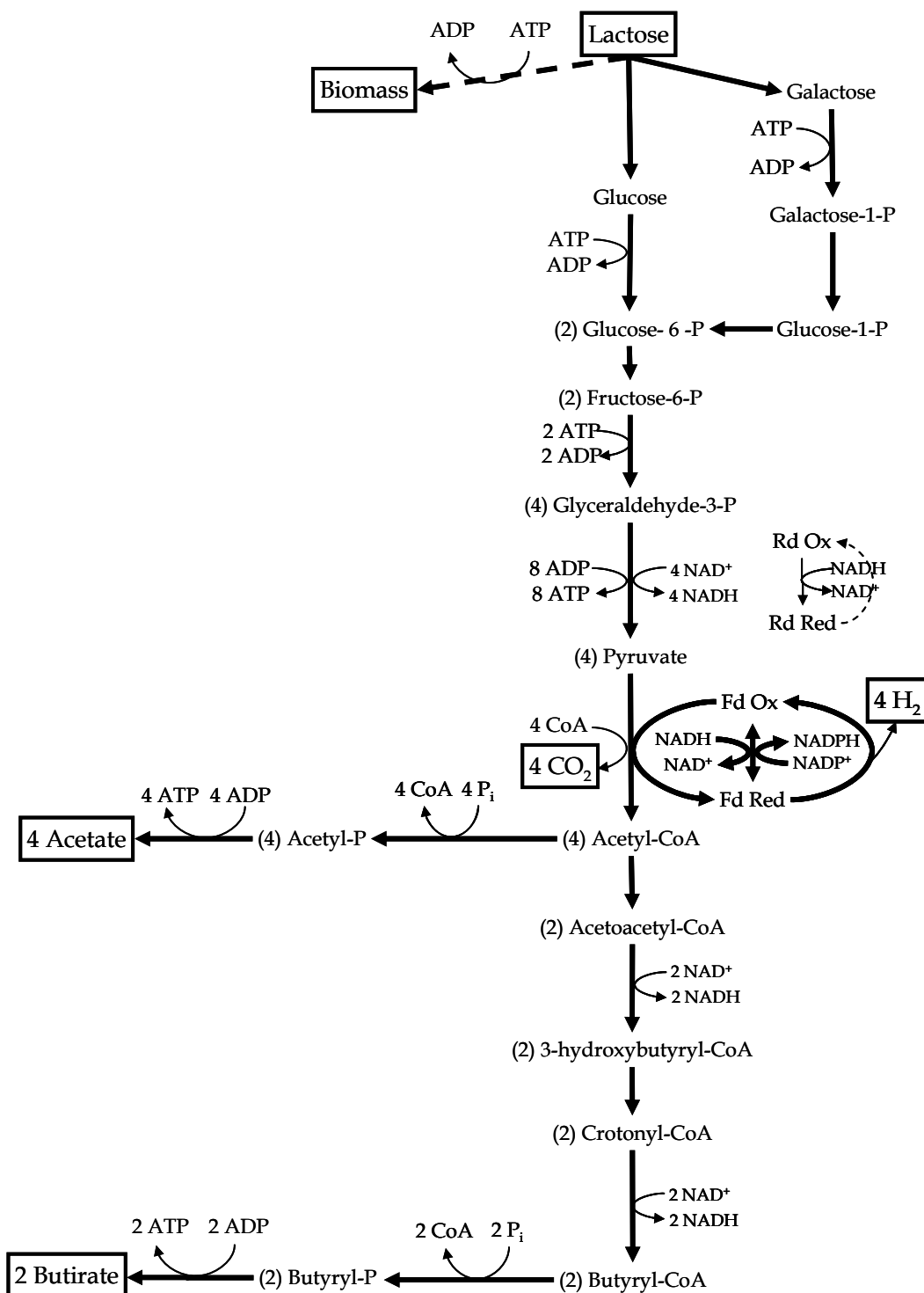


Figure 1.1 Metabolic pathway of Lactose by C acetobutylicum, under acidogenic phase, in box extracellular species

transferase; iii) glucose 6-phosphate by *phosphoglucomutase* is obtained before to start with the EMP pathway to produce pyruvate.

Comparing the oxidization steps of both the glucose and the galactose to the pyruvate it results that the two pathways are equivalent in terms of mass balance and energy balance. Therefore, the bioconversion of lactose may be considered corresponding to the conversion of two molecules (mole) of glucose. The contribute of the glycolytic step to the both the mass and energetic balances is: for one mole of lactose consumed along the EMP pathway, 4 moles of pyruvate and 4 moles of ATP are produced and 4 moles of NAD^+ are reduced to NADH_2 .

The successive steps of the overall pathway are hereafter.

The pyruvate reacts with the CoA and is cleaved in Acetyl-CoA by *pyruvate-ferredoxin oxidoreductase*. For a mole of pyruvate, a mole of CO_2 is produced and a mole of Fd_{OX} is reduced to Fd_{RED} . At the same time, Fd_{RED} is re-oxidized in agreement with two possible paths. For a mole of Fd_{RED} : i) a mole of molecular hydrogen is produced by *hydrogenase*; ii) a mole of $\text{NAD}^+/\text{NADP}^+$ is reduced by *NADH-ferredoxin oxidoreductase/NADPH-ferredoxin oxidoreductase*.

The fate of the acetyl-CoA may be twofold:

- A.1) A mole of acetyl-CoA adds a mole of phosphate by *phosphotransacetylase*, forming acetyl-phosphate that is subsequently converted in acetic ac. by *acetate kinase* producing a mole of ATP.
- A.2) The *thiolase* catalyses the conversion of acetyl-CoA (2 mole) in CoA (1 mole) and acetoacetyl-CoA (1 mole). The *3-hydroxybutyryl-CoA dehydrogenase* catalyzes the reduction of acetoacetyl-CoA to 3-hydroxybutyryl-CoA, coupled with the oxidization of 1 mole of NADH_2 for mole of acetoacetyl-CoA. Then, *crotonase* catalyzes the dehydration of 3-hydroxybutyryl-CoA to crotonyl-CoA, which in its turn is further reduced to butyryl-CoA by *butyryl-CoA dehydrogenase* (with the oxidation of 1 mole of NADH_2 for mole of crotonyl-CoA).

The fate of the butyryl-CoA is similar to that of the acetyl-CoA with respect to the formation of the acetic ac.:

- A.3) the combined action of *phosphate butyltransferase* and *butyrate kinase* convert butyryl-CoA in butyric ac. with the production of 1 mole of ATP for 2 moles of acetyl-CoA converted.

Solventogenic phase

Figure 1.2 reports the metabolic pathways of lactose by *C. acetobutylicum* under solventogenic phase. The cells are able to metabolize acids as well as lactose to produce solvent.

The lactose is converted in acetyl-CoA in agreement with the EMP pathway operating under the acidogenic phase.

Acetyl-CoA and butyryl-CoA may also be formed by the re-conversion of acetic and butyric acids, apart from the paths A.1) and A.4) recalled above. In particular four pathways may be identified:

- S.1) *acetoacetyl-CoA:acetate/butyrate:CoA transferase* catalyses the conversion of equimolar amount of acetic ac. and acetoacetyl-CoA to acetyl-CoA and acetoacetate (molar ratio 1:1);
- S.2) *acetoacetyl-CoA:acetate/butyrate:CoA transferase* catalyses the conversion of equimolar amount of butyric ac. and acetoacetyl-CoA in butyryl-CoA and acetoacetate (molar ratio 1:1);
- S.3) Assuming that enzymatic reactions are reversible, the butyryl-CoA - formed by acid reversion - may be re-converted in acetyl-CoA, in agreement with the

inverse mechanism with respect to its formation from acetyl-CoA;

S.4) the acetoacetate produced during the pathways S.1) and S.2) is cleaved in acetone and CO₂ by *acetoacetate decarboxylase*.

Noteworthy, the pathways S.1) through S.4) do not require energy (ATP). On the other hand, the reverse mechanism of A.1) and of A.3) requires energy, therefore the pathway is thermodynamically unfavorable. Hartmanis *et al.* (1984) observed that the activity assessed during the acidogenesis phase of *phosphate acetyltransferase*, *phosphate butyryl-transferase*, and *acetate kinase* rapidly decreased at the solventogenesis onset. On the basis of these findings, the author suggested that during the solventogenesis phase the acids are not uptake by the inverse pathways A.1) and A.3), or at least the uptake due to these pathways is negligible.

The fate of the acetyl-CoA may be twofold:

- it can be reduced to ethanol by the sequential action of *acetaldehyde dehydrogenase* and *ethanol dehydrogenase*. As a whole, one mole of acetyl-CoA oxidizes two mole of NADH₂, and produces one mole of ethanol.
- it can be converted in butyryl-CoA, in agreement with the pathway A.2)

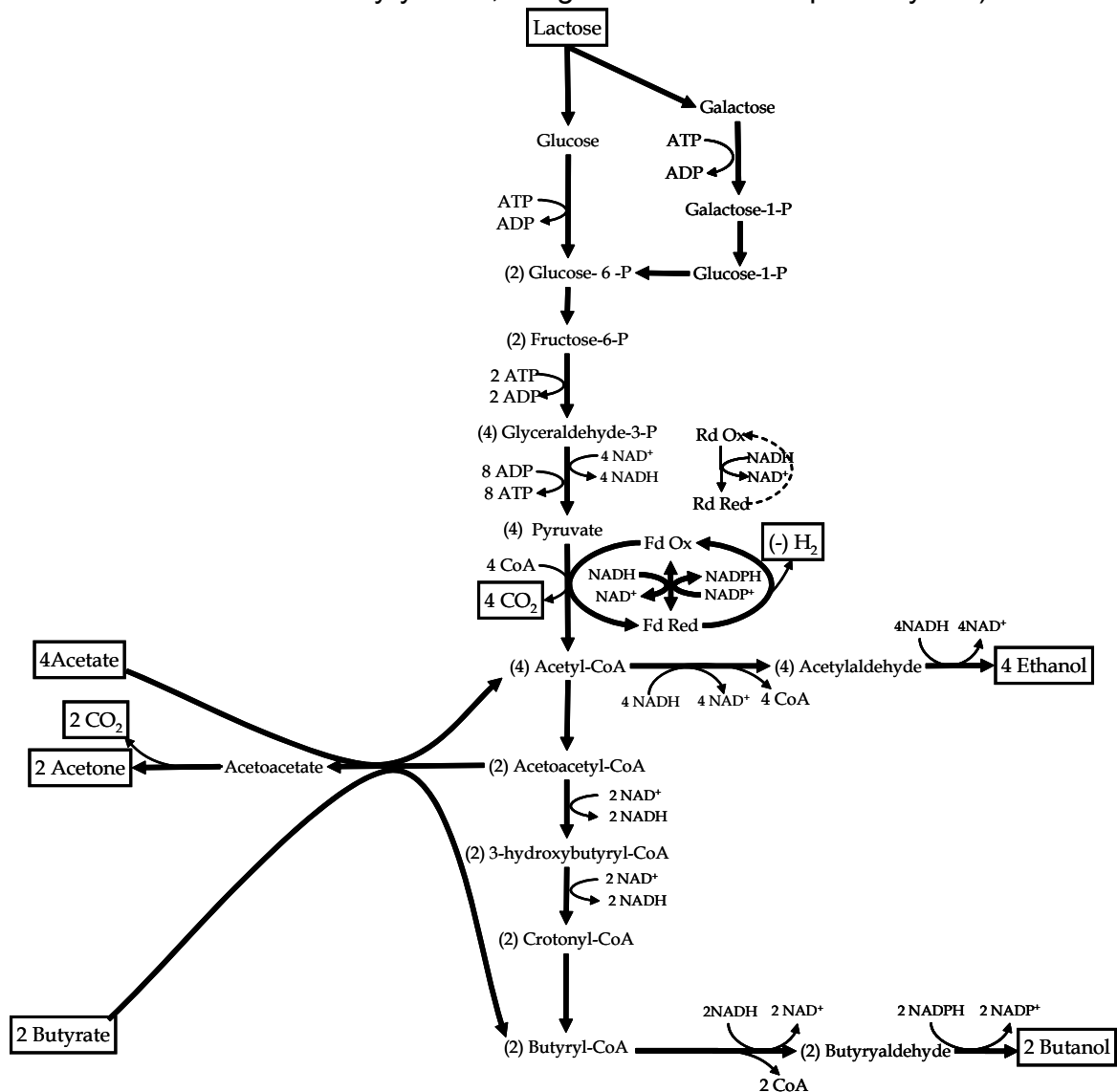


Figure 1.2 Metabolic pathway of Lactose by *C. acetobutylicum*, under solventogenesis phase, in box words are referred to external compound

The butyryl-CoA is reduced to butanol by the sequential action of *butyraldehyde dehydrogenase* and *butanol dehydrogenase*. As a whole, one mole of butyryl-CoA oxidizes two mole of NADH₂, and produces one mole of butanol.

The above pathway highlights that the acetone production is coupled with acids reconversion. Hartmanis *et al.* (1984) worked out data available in literature on the molar ratio between the acids uptake and the acetone produced. The mean value of the ratio was 0.97 (with a standard deviation of 0.11) and he suggested that the production of acids is coupled with the formation of equimolar amounts of acetone.

1.4 Stoichiometry

The stoichiometric equations referred to the lactose conversion process may be obtained by summing up the single enzymatic steps involved in the production of the biomass and of the metabolites. Knowing all the reaction steps, the relationships among the amount of substrate converted, intracellular/intermediate metabolites produced (e.g.: pyruvate, acetyl-CoA...), extracellular metabolites produced and biomass produced may be assessed.

The relationships among the species involved in the fermentation process may be assessed by working out both the mass balance referred to the intermediate species (e.g., pyruvate, acetyl-CoA, ...) and the balance referred to the biosynthetic (ATP) and/or to the reducing agents (NADH₂, Fd_{RED},). It is possible to assume that some species are under steady state conditions and their concentration may be assumed constant. As a consequence, the pathway steps involving these peculiar species may be summed up and an overall reaction may be proposed for the material balance on the relevant species.

The balance on the reduction energy is extended to all enzymatic reactions characterized by a net reaction energy and to the reactions in charge for the transformation of the energy in its various forms (NADH₂, NADPH₂, FdH₂ ...). The balance on reducing agents - expressed in its various forms - is crucial for the

Table 1.1. Formation reactions of biomass and metabolites

Acidogenesis phase	
$C_{12}H_{22}O_{11} \cdot H_2O + 3NH_3 + 3 \frac{MW_x}{Y_{ATP}} ATP \rightarrow 3C_4H_7O_2N + 6H_2O$	(T.1.1.1)
$C_{12}H_{22}O_{11} \cdot H_2O + 4H_2O \rightarrow 8ATP + 4C_2H_4O_2 + 4CO_2 + 8H_2$	(T.1.1.2)
$C_{12}H_{22}O_{11} \cdot H_2O \rightarrow 6ATP + 2C_4H_8O_2 + 4CO_2 + 4H_2$	(T.1.1.3)
Solventogenesis phase	
$C_{12}H_{22}O_{11} \cdot H_2O \rightarrow 4ATP + 4C_2H_6O + 4CO_2$	(T.1.1.4)
$C_{12}H_{22}O_{11} \cdot H_2O + 2C_2H_4O_2 \rightarrow 4ATP + 2C_2H_6O + 2C_3H_6O + 6CO_2 + 4H_2$	(T.1.1.5)
$C_{12}H_{22}O_{11} \cdot H_2O + 2C_4H_{10}O_2 + 2H_2O \rightarrow 4ATP + 4C_2H_6O + 2C_3H_6O + 6CO_2 + 6H_2$	(T.1.1.6)
$C_{12}H_{22}O_{11} \cdot H_2O \rightarrow 4ATP + 2C_4H_{12}O + 4CO_2$	(T.1.1.7)
$C_{12}H_{22}O_{11} \cdot H_2O + 2C_2H_4O_2 \rightarrow 4ATP + C_4H_{12}O + 2C_3H_6O + 6CO_2 + 4H_2 + H_2O$	(T.1.1.8)
$C_{12}H_{22}O_{11} \cdot H_2O + 2C_4H_{10}O_2 \rightarrow 4ATP + 2C_4H_{12}O + 2C_3H_6O + 6CO_2 + 4H_2 + H_2O$	(T.1.1.9)

soundness of the fermentation model for butyric-acid bacteria. It should be noted that the conservation of biosynthetic energy (ATP) is made troublesome by the lack of information about the biomass growth and maintenance, two processes that uptake ATP at a rate depending on the operating conditions.

With reference to the conversion of lactose by *C. acetobutylicum*, the elemental material balances are hereafter reported, assuming that all intracellular species are under pseudo steady states. The balances are referred to Carbon (C), Hydrogen (H), Oxygen (O) and Nitrogen (N).

The redox balance may be conveniently written in terms of the reductance degree (γ), defined as the number of equivalents of available electrons per atom of carbon in the compound (Papoutsakis, 1984). For the generic compound $C_xH_yO_zN_k$ reductance degree is defined as $(4X+Y-2Z-3K)/X$. In agreement with the definition, the reductance degree of formaldehyde (CH_2O) is 4, and it is zero for water (H_2O) and ammonia (NH_3). The reductance degree of species characterized by elemental composition $n-(CH_2O)$ is still 4.

Acidogenic phase

Equations (T.1.1.1) through (T.1.1.3) of table 1.1 report the formation reactions of biomass and metabolites. For sake of simplicity, the ADP has been omitted where present.

With reference to the biomass in eq. (T.1.1.1), the biomass has been described as $C_4H_7O_2N$ and is characterized by: molecular weight $MW_X=101$ g/mol; carbon fraction $\alpha=0.475$; reductance degree $\gamma=4$. The elemental composition of the biomass has been selected in agreement with a previous work carried out by Zeng *et al.* (1996) for *C. butylicum*. It should be highlighted that the elemental composition of the microorganism reported in the literature ranges over a quite wide range. In particular, Papoutsakis (1984) and Andrews (1989) have suggested that γ and α , two important biological regularities, are typically equal to 4.262 ± 0.172 and 0.462 ± 0.023 , respectively. As a consequence of the values of γ and α adopted in eq. (T.1.1.1), the formation of the biomass does not require the oxidation of $NADH_2$.

The analysis of eq. (T.1.1.2) shows that for one mole of lactose converted in acetic ac. 8 moles of ATP are produced: 4 moles during glycolytic pathway, and 4 moles during the conversion of acetyl-CoA to acetic ac. Likewise, for one mole of lactose converted in butyric ac. 6 moles of ATP are generated: 4 moles during glycolytic pathway, and 2 during conversion of butyryl-CoA to butyric ac..

In agreement with the assumption of absence of $NADH_2$ oxidation during biomass formation, an energy accumulation (in the form of $NADH_2$) would be expected coupled with acids production although the assumption of pseudo steady state. The excess of $NADH_2$ may be oxidized by *ferredoxin oxidoreductase cycle* (fig. 1) producing molecular hydrogen.

The soundness of the selection of the network of formation reaction reported in table 1.1, the assumptions of $\gamma=4$, and that $NADH_2$ in excess produces molecular hydrogen is supported by the study carried out by Rogers (1984). Under acidogenic phase, he reported a molar ratio butyric/acetic acids equals to 1.5, and a ratio H_2/CO_2 equals to 1.25, in agreement with Eq.s (T.1.1.1), (T.1.1.2) and (T.1.1.3).

The biochemical energy (ATP) accumulated during the acids formation fuels the biomass formation (eq. 1). Therefore, to write down the energy balance is necessary to know the ATP yield of eq. (T.1.1.1), which varies with the operating conditions.

Solventogenic phase

Equations (T.1.1.4) through (T.1.1.9) of table 1.1 describe the production of ethanol and butanol from lactose, directly and *via* acids uptake.

The comparison of eq.s (T.1.1.1)-(T.1.1.3) with eq.s (T.1.1.4)-(T.1.1.9) shows that the ratio between molecular hydrogen produced and lactose consumed decreases moving from acidogenesis to solventogenesis phases. The decrease is due to larger amount of reducing energy required for solvents production with respect to the acids productions.

The analysis of table 1.1 points out that, except for the production of ethanol from butyric ac. (eq. T.1.1.6), the production of solvents by reconversion of acids is associated with the production of four moles of molecular hydrogen for mole of lactose converted (table 1.1, eq.s 5, 8 and 9). The ethanol production by butyric ac. is associated with the production of six moles of hydrogen for mole of lactose converted. On the other hand, Woods (1945) highlighted that the largest part of the butyric ac. uptake is coupled with the butanol production (Woods, 1945). Moreover, more than 55% of the acetic ac. is converted to butanol, while the largest part of the remaining fraction is decarboxylated to acetone and carbon dioxide. Altogether, results available in literature allow neglecting the relevance of eq. (T.1.1.6) in the ABE fermentation.

In agreement with table 1.1, the production of ATP associated to the solvent production (4 moles of ATP for mole of lactose consumed) should accumulate in the solventogenic cells. On the hand, steady states assumption adopted for intracellular metabolites asks for ATP uptake. Since solventogenic phase is characterized by the absence of cell growth (ATP consuming), the ATP produced should be consumed in the solventogenic biomass maintenance.

Working out eq.s in table 1.1, the range of the fractional yield of lactose to butanol ($Y_{B/L}$) may be assessed. The maximum theoretical $Y_{B/L}$ is 0.41g/g is obtained by both re-conversion of the butyric ac. eq. (T.1.1.9), and direct conversion of lactose eq. (T.1.1.7). The $Y_{B/L}$ drops to half the maximum when butanol is produced via acetic ac. re-conversion eq. (T.1.1.8).

Even though the two pathways eq.s (T.1.1.7) and (T.1.1.9) are equivalent in terms of $Y_{B/L}$, they differ in terms of butanol selectivity, being larger for eq. (T.1.1.7). The performance of the ABE process may improve by increasing the relevance of the direct conversion of lactose in butanol with other routes.

1.5 The New Butanol Enterprises

In the last decade great hope have been reposed in “white biotechnology”, from West to East, and in its ability to produce in the next future economically competitive chemical bulk. The huge capital invested in “white biotechnology” research by several large companies is the witness of the new wind. Recently Bp and DuPont in partnership have created the “BP Energy Biosciences Institute” at University of California (Berkeley) with an endowment of 500 M US\$ over 10 years, and similar investments are made by other companies in unison with government agencies to promote the parallel development of bio-based research in academia and in the industry. Similar events are happening in China as well as in India. Where the high ranking politicians, the general public and the large companies as well as University are much more eager to embark on a journey towards new horizons. Unfortunately, in Italy is not so.

Although great improvement have been reached by molecular biology techniques with engineered microorganism to increase productivity, yields and

selectivity (Papoutsakis, 2008), lack of equipments innovation may be discovered with a negative spin-off for the economic feasibility of processes on large scale. In particular, the information regarding the use of new reactor and the strategies to optimize the process are not based on a systematic kinetic study but rather than on good results of tests, or instinctive idea (Villadsen, 2007).

The information regarding the kinetic characterization and the development of model that permit optimization of process through the classic methods of Chemical Engineering are still lacking, to confirm this opinion recently Villadsen (2007) reports that recent literature on the “new technology” for the ABE process shows that most of the advances were made by serendipity rather than by application of “fundamental” science, and emphasizes that application of classical engineering principles can lead to technological advances on par with those obtained in molecular biology.

1.6 Biofilm reactor

The relevance of the biofilm reactor in the ABE production process has been highlighted in § 1.2. In agreement with Maddox (1989) and Qureshi and Blaschek (2001) the selection of the optimized reactor configuration should take into account the specific production rate and the operating simplicity should. In particular, for a biofilm reactor the specific production rate may be increased with the biofilm concentration. Therefore, particular attention must be paid at the biofilm formation, growth, detachment and stability.

Cells may be immobilized by three different techniques (see Russo, 2008, for more details): adsorption, entrapment, and covalent bond formation. The entrapment and covalent bond techniques require chemicals that may restrict propagation/increase cell concentration in the reactor and it may increase the process investments. The third technique – a natural process – consists in “adsorption/adhesion” of cells to supports and growth of firm biofilm (Tyagi and Ghose, 1982; Forberg and Haggstrom, 1985; Qureshi and Maddox, 1987). The latter technique is widely adopted in processes characterized by huge throughput of inexpensive products (Cooper and Atkinson, 1981; Crueger and Crueger, 1989; Nicoletta *et al.*, 2000; Aivasidis and Diamantis, 2005).

In nature biofilms exist primarily as complex multi-species communities of bacteria in which each species fills an ecological niche within the biofilm depending on its metabolism and morphology (Stoodley *et al.*, 2002). The nature of mixed culture biofilms is dependent on which species are present and what role each species fills. Multi-species biofilms are adopted industrially to achieve several aims, including the treatment of wastewaters for removal of organics (Taras *et al.*, 2005) and heavy metals (Meyer and Wallis, 1997). The presence of multiple species allows for the treatment of waste streams that are diverse in composition and that fluctuate in component concentration.

Single species biofilm are adopted industrially to produce chemicals (Meyer and Wallis, 1997).

Biofilms are adopted in various types of reactors such as continuous stirred tank reactors (CSTRs), packed bed reactors (PBRs), fluidized bed reactors (FBRs), airlift reactors (ALRs), upflow anaerobic sludge blanket (UASB) reactors, and expanded granular sludge bed (EGSB) reactors etc. (Demirci and Pometto, 1997; Lettinga *et al.*, 1980, 1983; Seghezzo *et al.*, 1998).

1.6.1 Biofilm Formation

A biofilm is defined as a structured community of bacterial cells enclosed in a

self-produced polymeric matrix and adherent to an inert or living surface (Costerton *et al.* 1999). In general, a mature biofilm develops through four stages: initial attachment, irreversible attachment by the production of extracellular polymeric substances (EPS), early development, and maturation of biofilm architecture (Stoodley *et al.*, 2002).

The life of a biofilm starts with the planktonic or free floating cells (fig. 1.3). Surfaces immersed in an aqueous solution usually become charged and attract/concentrate inorganic solutes and charged or highly polar organic molecules. The concentration of cations, glycoproteins, proteins, and organic molecules at the surface provide a favourable region for bacteria growth, when compared to the bulk aqueous environment (Annachatre and Bhamidimarri, 1992). In addition, fluid flow stress in the boundary region near the surface may be considered negligible allowing bacteria to approach the surface. Cells flows towards the surface and form a temporary association with the surface or microbes already present on the surface (Watnick and Kolter, 2000). Then, planktonic bacterial cells may dissociate from the surface and resume the planktonic state or become irreversibly attached to the surface with the production of EPS.

The EPS layer has several functions: i) binding the cell to the surface; ii) protection of the cells from the mechanical stresses; iii) protection of the cells with respect toxic compounds that could harm; iv) barrier to phagocytes and bactericides. A disadvantage, the EPS represents a barrier to nutrients necessary for cell growth making the microenvironment in the biofilm strongly different from the conditions stabilized in the liquid bulk. As a result, the biofilm performances depend on a delicate equilibrium among several phenomena acting on its structure: biofilm detachment, biofilm sloughing, biofilm growth, etc.

Biofilm detachment

Detachment is the loss of biomass or individual cells from a mature biofilm. Several models are available about the rate of biofilm detachment. Applegate and Bryers (1991) reported that the growth conditions of the biofilm strongly affect biofilm conversion processes.

The biofilm detachment process may be controlled by cell physiology, rather than by hydrodynamics. Boyd and Chakrabarty (1994) suggested that biofilm detachment process could be genetically controlled.

Biofilm sloughing

The catastrophic loss of pieces or part of biofilm is called sloughing. At laboratory scale tests, sloughing phenomena may determine the end of a test. At the

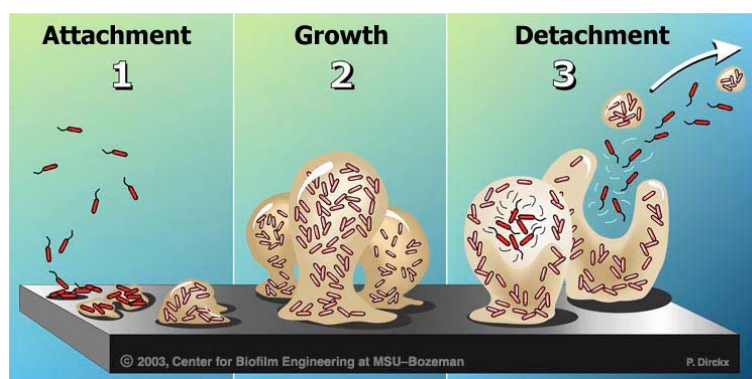


Figure 1.3 Sketch of biofilm formation on a flat surface under stagnant conditions.
Available on-line on <http://www.erc.montana.edu>

industrial scale, sloughing is associated with intolerably excursion of the free biomass concentration in bioreactor effluents.

The main responsible agent of the sloughing is the hydrodynamic of the reactor investigated.

Factors enhancing biofilm formation

Several parameters affect how quickly biofilms form and mature, including surface, cellular, and environmental factors. The surface onto which cells adhere has a relevant impact on biofilm formation. Rough surfaces tend to enhance biofilm formation (Characklis *et al.*, 1990). Shear stresses are low near a rough surface. Porous materials also work well for biofilm formation: shear stresses are negligible inside pores even in turbulent reactors. Pores provide a protected environment for cells to attach and grow. Porous materials such as brick and bonechar have been used to immobilize *Clostridium* cells used in biofilm reactors (Qureshi *et al.*, 2004).

Biofilm formation increase with the hydrophobicity of the surface material (Donlan and Costerton, 2002). Biofilms form much more rapidly on Teflon and other plastics than glass or metal. Support features that enhance biofilm formation are summarized in Table 1.2.

The concentration of substrates in the medium may affect the rate of biofilm formation. Biofilms tend to form more readily in the presence of ample nutrients (Cowan *et al.*, 1991).

High temperatures may enhance biofilm formation. Depending on the strains, high temperature increases the rate of cell growth, EPS production, and surface adhesion. Features that enhance biofilm formation (Annachatre and Bhamidimarri, 1992).

1.6.2 Biofilm reactors

The spectrum of biofilm reactor typologies has been reported by Olivieri *et al.* (2009). Basically, the reactor configurations include: continuous mechanically agitated tank (CSTR), packed bed (PBR), trickling bed (TBR), fluidized bed (FBR), airlift reactors (ALR), upflow anaerobic sludge blanket (UASB), and expanded bed reactors. Operating conditions depends on the reactor typology.

PBRs are packed with suitable support material. Provided the formation of the biofilm, the reactor is operated under continuous conditions. Depending on the strain, substrates, and support, the biofilm formation may take from a few to several days. Reactors are usually fed at the bottom, getting products at the top. These reactors may be easily clogged as a consequence of the excessive cell growth. In *C. acetobutylicum*/*C. beijerinckii* biofilm packed bed reactors, reaction rates up to 45 times that of the batch (control) reactors have been obtained (Qureshi *et al.*, 2000).

Table 1.2. Effects of support features on biofilm formation.

Support characteristic	Effects
Surface tension	System dependent
Surface charge	System dependent
Porosity	No effect
Surface roughness	Increase cells attachment
Hydrophobicity	Increase cells attachment
Wettability	Increase cells attachment
Surface area	No effect

Table 1.3. Production of ABE in packed bed reactor.

Strain/Support	Maximum solvent (g/L)	Maximum productivity (g/Lh)	Biomass accumulated (g _{DM} /L)	Biomass accumulation (g _{DM} /g _{support})
<i>C. acetobutylicum</i>				
Bonechar	9.3 (0.30)	6.50 (1.5)	74.0	0.087
Glass beads	3.0 (0.31)	0.93 (0.31)	65.0	0.044
Glass wool	3.0 (0.10)	0.30 (0.10)	3.1	0.050
Polypropylene	2.3 (0.25)	0.58 (0.25)	0.8	-
Stainless steel balls	2.0 (0.07)	0.15 (0.07)	1.0	-
<i>C. beijerinckii</i>				
Clay brick	7.9 (2.0)	15.8 (2.0)	73.7	0.093

TBRs are similar to the PBRs except from the feeding point: the substrate bearing stream is fed at the top. A disadvantage of these reactors is the possibility that biofilms may not get sufficient substrates with a reduction of the process performances.

FBRs have played a successful role in the degradation of toxic phenolic chemicals (Woolard and Irvine, 1994; Wobus *et al.*, 1995; Veeresh *et al.*, 2005) and butanol production (Qureshi and Maddox, 1991; Qureshi and Maddox, 1988). In these reactors, cell growth occurs around the adsorbent particles. Formation of active biofilms around the particles and accumulation of sufficient biomass in the reactor may take from 2 to 4 weeks. A major advantage in these reactors is that they can be operated for much longer periods than PBR. Clogging is prevented by the shear stresses. Studies available on butanol production report productivity approximately 40–50 times that of the batch reactors.

Biofilm reactors for butanol production

Cells adsorption is typically adopted to promote biofilm formation. Typically, cells of *C. acetobutylicum* are inoculated in reactors packed with adsorption supports. The adsorption process varies from 2–3 days to weeks depending on the strain, supports, substrates, and the reactor configurations. Forberg and Haggstrom (1985) adopted beechwood shavings as biofilm supports for *C. acetobutylicum* in a reactor fed continuously with a glucose solution. An active biofilm was formed on the wood shavings and a reactor productivity as high as 1.53 g/hL was measured. Later, Qureshi and Maddox (1987) reported a production of butanol in biofilm (bonechar supported) reactor of *C. acetobutylicum* from whey permeate of 4.5 g/hL. Moreover, he noted that biofilm formation on this support was quick.

Welsh *et al.* (1987) investigated a number of supports for butanol production by *C. acetobutylicum* in batch and continuous systems. The supports investigated were coke, kaolinite, and Gel White (a montmorillonite clay). Coke was reported to be superior to other supports. The maximum concentration of ABE in the reactor effluent was 12 g/L at dilution rate of 0.1 h⁻¹, productivity of 1.2 g/hL.

Studies on butanol production by means of biofilm of *C. acetobutylicum* pointed out the formation of a visible biofilm layers in 2–4 days (in packed bed reactors), and that the butanol production begin after 4th day of continuous operation (Qureshi *et al.*, 2005). Several supports were investigated and reactor performance are reported in table 1.3

1.7 Scope 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 Department of Chemical Engineering of the University of Naples 'Federico II'. The activities were articulated along three paths: i) the characterization of the ABE fermentation process as regards kinetics and yields; ii) the development of a continuous biofilm reactor for the ABE production; iii) the economic assessment of the process for the recover and concentration of solvents.

A commercial *clostridia* strain was investigated. *Clostridium acetobutylicum* DSM 792 was selected for its ability to produce ABE with satisfactory selectivity towards the butanol. Besides the specific fermentation process investigated, the methodology and strategy developed in the present program propose as a tool for anaerobic fermentation investigation.

The tests were carried out with lactose solutions to mime the cheese whey, a lactose-rich wastewater which is an abundant byproduct of the dairy industry. From the substrate point of view the investigated process proposes as a strategy to exploit a wastewater particular expensive to remediate for the high COD.

Characterization of the ABE fermentation process as regards 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 lactose fermentation by *C. acetobutylicum*. The study regarded both experimental and theoretical aspects of the fermentation.

The investigation was carried out with batch and continuous cultures of free *C. acetobutylicum* cells. Batch tests were focused on the preliminary characterization of the fermentation with the aim of highlighting the relevant features of the process. Tests carried out with a CSTR were aimed to the systematic characterization.

Continuous cultures were carried out under a wide interval of operating conditions in order to characterize the fermentation process with regards to both the acidogenesis and the solventogenesis phases. 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. Those with the second were designed for the characterization of the solventogenesis phase. The fermentation tests were characterized in terms of cells, metabolites and lactose concentrations.

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

Development of a continuous biofilm reactor for the ABE production.

The study was aimed at the optimization of the cells immobilization process and at the assessment of the butanol production by means of *C. acetobutylicum* biofilm.

The immobilization process was optimized with reference to the granular supports. The activities were focused on the selection of the carrier that maximizes the biofilm formation.

The activity aimed at the butanol production regarded the design, set-up and operation of a biofilm fixed bed reactor. Operating conditions of the continuous tests

were selected to maximize the butanol production and butanol selectivity.

Economic assessment of the process for the recovery and concentration of solvents.

The study was aimed at investigating the economical feasibility of butanol recovery from ABE fermentation by means of an unconventional flowsheet.

The activities were focused on the selection of unit operations, their simulation by means of the Aspen Plus® process simulator. The flowsheet was optimized assuming the venture profit as objective function.

The thesis will separately address the three topics. Section 2 proposes a theoretical framework of the investigated process. Section 3 and 4 regards the fermentation characterization. Section 5 addresses the butanol productions in a biofilm reactor. Section 6 reports on the economic assessment of the recovery and concentration process. Within each section manuscripts submitted to scientific journals and pertinent to the topic are included.

2 THEORETICAL FRAMEWORK

The *clostridia* fermentation under solventogenic conditions is characterized by the contemporary production of acids and solvents as well as by the co-presence of three types of cellular morphologies: i) high motile cells, that produce acids and are able to multiply; ii) endospore able to produce solvent but unable to multiply; iii) spore unable to metabolize substrate and to multiply. This scenario of products and cellular morphologies makes the kinetic characterization of the process very complex. Therefore, a proper strategy should be designed to isolate single issues of the fermentation process to integrate results in a detailed model.

The analysis of freedom degrees of the system based on mass and energetic balances allows to single out the key variables of the process. In this section the potential set of equations describing the investigated fermentation process are listed and analysed in order to select the key variables.

Metabolites balance

Table 2.1 summarizes the reactions of the fermentation process as regards both acidogenic and solventogenic stage.

Table 2.2 reports molar balance referred to extracellular liquid metabolites extended to the reaction volume unit. In the table, f_n is the molar rate of

Table 2.1. Possible metabolic reaction during *clostridia* fermentation on lactose (L, lactose; AA, acetic acid; BA, butyric acid, B, butanol, Et, ethanol; Ac, acetone)

Acetic ac.	$1/2L \rightarrow 4 \text{ ATP} + 2\text{AA} + 2\text{CO}_2 + 4\text{H}_2 - 2\text{H}_2\text{O}$	(T.2.1.1)
Butyric ac.	$1/2L \rightarrow 3\text{ATP} + \text{BA} + 2\text{H}_2 + 2\text{CO}_2$	(T.2.1.2)
Ethanol and acetone	$L + 2\text{AA} \rightarrow 4\text{ATP} + 6 \text{CO}_2 + 4\text{H}_2 + 2\text{Et} + 2\text{Ac}$	(T.2.1.3)
Butanol and acetone	$L + 2\text{AA} \rightarrow 4\text{ATP} + 6\text{CO}_2 + 4\text{H}_2 + \text{H}_2\text{O} + 2\text{Ac} + \text{B}$	(T.2.1.4)
Butanol and acetone	$L + 2\text{AB} \rightarrow 4\text{ATP} + 6\text{CO}_2 + 4\text{H}_2 + 2\text{Ac} + 2\text{B}$	(T.2.1.5)
Butanol	$L \rightarrow 4\text{ATP} + 4\text{CO}_2 + 2\text{H}_2\text{O} + 2\text{B}$	(T.2.1.6)
Ethanol	$L \rightarrow 4\text{ATP} + 4\text{CO}_2 + 4\text{Et}$	(T.2.1.7)
Biomass	$L + 3\text{NH}_3 + 3(101/Y_{\text{ATP}})\text{ATP} \rightarrow 3\text{X}$	(T.2.1.8)

Table 2.2 Molar balance on extracellular liquid metabolites of ABE fermentation extended to the unit reaction volume (legend as table 2.1)

Lactose	$F_L = \frac{F_X}{3} + \frac{f_1^{\text{AA}}}{4} + \frac{f_2^{\text{BA}}}{2} + \frac{f_6^{\text{B}}}{2} + \frac{f_7^{\text{Et}}}{4} + \frac{f_3^{\text{Ac}}}{2} + \frac{f_4^{\text{Ac}}}{2} + \frac{f_5^{\text{Ac}}}{2}$	(T.2.2.1)
Acetic ac.	$F_{\text{AA}} = f_1^{\text{AA}} - f_3^{\text{AA}} - f_4^{\text{AA}}$	(T.2.2.2)
Butyric ac.	$F_{\text{BA}} = f_2^{\text{BA}} - f_5^{\text{BA}}$	(T.2.2.3)
Butanol	$F_{\text{B}} = f_4^{\text{B}} + f_5^{\text{B}} + f_6^{\text{B}}$	(T.2.2.4)
Ethanol	$F_{\text{Et}} = f_7^{\text{Et}} + f_3^{\text{Et}}$	(T.2.2.5)
Acetone	$F_{\text{Ac}} = f_3^{\text{Ac}} + f_4^{\text{Ac}} + f_5^{\text{Ac}}$	(T.2.2.6)

production/uptake of the species “i” referred to the equation (T.2.2.n), F_i the molar net rate of production/uptake of the species “i” assessed experimentally.

Table 2.3 reports stoichiometric relationships from the reaction set reported in table 2.1. The relationships are then adopted to reduce the complexity of the equations set in table 2.2 and a simplified set of 6 equations are reported in table 2.4. Particular attentions should be paid at the productivity and selectivity of butanol that are related to the value of the butanol production rate f_6^B .

The rank of the equations system in table 2.4 is 5: this means that 5 of the 6 equations are linearly independent. The balance on lactose, in fact, may be obtained as linear combination of the other equations. The sixth equation may be adopted to assess the accuracy of the results. In particular, the comparison between the experimental and theoretical values of the lactose consumed may be adopted as a measure of the results accuracy.

The equation system in table 2.4 results of 5 equations in 7 variables (f_1^{AA} , f_2^{BA} , f_3^{AA} , f_4^{AA} , f_5^{BA} , f_6^B , f_7^{Et}). Some considerations must be done to make the system determined.

Results reported in literature, and confirmed in the present investigation (see § 4.3), have pointed out that the production rate of the ethanol is usually lower than that of the butanol. Accordingly, the analysis of the routes to produce ethanol suggests that: i) the amount of acetone produced during acetic acid conversion to ethanol is very low (eq. T.2.1.3) with respect the other paths; ii) the lactose consumed to produce ethanol is a small fraction of the overall lactose converted.

Table 2.3 Stoichiometric relationships among extracellular liquid metabolites of the ABE fermentation from reaction in table 2.1.

$f_3^{Ac} + f_4^{Ac} + f_5^{Ac} = f_3^{AA} + f_4^{AA} + f_5^{BA}$	(T.2.3.1)
$f_4^{Ac} = f_4^{AA}$	(T.2.3.2)
$f_5^{Ac} = f_5^{BA}$	(T.2.3.3)
$f_5^B = f_5^{BA}$	(T.2.3.4)
$f_4^B = \frac{f_4^{AA}}{2}$	(T.2.3.5)
$f_3^{Et} = f_3^{AA}$	(T.2.3.6)

Table 2.4 Molar balance on external metabolites in function of acids and solvents directly produced from lactose

Lactose	$F_L = \frac{F_X}{3} + \frac{f_1^{AA}}{4} + \frac{f_2^{BA}}{2} + \frac{f_6^B}{2} + \frac{f_7^{Et}}{4} + \frac{f_3^{AA}}{2} + \frac{f_4^{AA}}{2} + \frac{f_5^{BA}}{2}$	(T.2.4.1)
Acetic ac.	$F_{AA} = f_1^{AA} - f_3^{AA} - f_4^{AA}$	(T.2.4.2)
Butyric ac.	$F_{BA} = f_2^{BA} - f_5^{BA}$	(T.2.4.3)
Butanol	$F_B = \frac{f_4^{AA}}{2} + f_5^{BA} + f_6^B$	(T.2.4.4)
Ethanol	$F_{Et} = f_7^{Et} + f_3^{AA}$	(T.2.4.5)
Acetone	$F_{Ac} = f_3^{AA} + f_4^{AA} + f_5^{BA}$	(T.2.4.6)

Moreover, the ATP produced according to the acetic ac. conversion to ethanol (eq. T.2.1.3) is equal to the ATP produced during direct conversion of lactose (eq. T.2.1.7) to ethanol. As a consequence, it is possible to suppose $f_3^{\text{Et}} + f_7^{\text{Et}} \approx f_7^{\text{Et}}$, that is the molar uptake rate f_3^{AA} is neglected in the system table 2.4 without any effects on the ATP balance.

Rodgers (1982) investigated the fermentation of *C. acetobutylicum* on glucose and pointed out that the molar ratio between butyric and acetic acids was constant and equal to 1.5 under acidogenic phase. Therefore, it is plausible that the investigated fermentation is still characterized by a constant molar ratio between butyric and acetic acids.

The analysis of reactions voted to produce/uptake the acids is now in order.

Under acidogenic fermentation it is possible to measure the ratio $f_2^{\text{BA}}/f_1^{\text{AA}}$ and the eq.s (T.2.4.1)-(T.2.4.2) yield:

$$F_{\text{AA}} = f_1^{\text{AA}} \quad (2.1)$$

$$F_{\text{BA}} = f_2^{\text{BA}} \quad (2.2)$$

Where F_{AA} and F_{BA} are assessed by experiments. As a consequence, it results:

$$\frac{f_2^{\text{BA}}}{f_1^{\text{AA}}} = \text{function}(\text{AA,BA,B,Ac,Et,L,pH}) \quad (2.3)$$

Table 2.5 summarizes the set of the six independent equations – eq. (2.3) and the set of equations linearly independent - in the six unknown (f_1^{AA} , f_2^{BA} , f_4^{AA} , f_5^{BA} , f_6^{B} , f_7^{Et}).

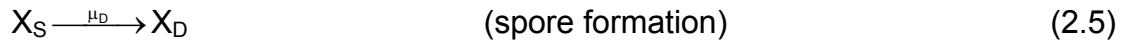
Biomass

Cells in the ABE fermentation process may be classified in three groups: acidogenic cells (concentration X_A), solventogenic cells (concentration X_S), and spore (concentration X_D). The assessment of the three concentrations allows to refer the specific butanol productivity only to the solventogenic cells.

The possible transformation paths of the microbial population under solvent production conditions are described by the eq. (T.2.1.8) and by the eq.s (2.4) through (2.6) (Sarrafzadeh *et al.*, 2005):

Table 2.5 Independent set of equations describing the external metabolites in the ABE process.

Acetic ac.	$F_{\text{AA}} = f_1^{\text{AA}} - f_4^{\text{AA}}$	(T.2.5.1)
Butyric ac.	$F_{\text{BA}} = f_2^{\text{BA}} - f_5^{\text{BA}}$	(T.2.5.2)
Butanol	$F_{\text{B}} = \frac{f_4^{\text{AA}}}{2} + f_5^{\text{BA}} + f_6^{\text{B}}$	(T.2.5.3)
Ethanol	$F_{\text{Et}} = f_7^{\text{Et}}$	(T.2.5.4)
Acetone	$F_{\text{Ac}} = f_4^{\text{AA}} + f_5^{\text{BA}}$	(T.2.5.5)
Butyric/acetic ac.s ratio	$\frac{f_2^{\text{BA}}}{f_1^{\text{AA}}} = \text{function}(\text{AA,BA,B,Ac,Et,L,pH})$	(T.2.5.6)



Where μ_S , μ_D , μ_{lyses} are the specific rate of solventogenic cells formation, spores formation and cell lyses, respectively. The latter has been assumed negligible under operating conditions investigated.

Table 2.6 reports the three mass balances on the biomass. In particular, W_X is the total biomass productivity, w_X^A , w_X^S and w_X^D the mass rate of production of acidogenic cells, solventogenic cells and spore, respectively.

In agreement with Bauchop and Elsdén (1960) it is possible to relate the biomass produced and the ATP booked for the growth (see § 4.2 for more details). They introduced the ATP yields (Y_{ATP}), expressed as dry mass of cells produce per mole of ATP generated. Under acidogenic conditions, the Y_{ATP} may be seen as a tool to link the acid production (eq.s T.2.1.1 and T.2.1.2) to the biomass growth (eq. T.2.1.8). Accordingly, it results:

$$X_A = \text{function}(Y_{\text{ATP}}, \mu, f_1^{\text{AA}}, f_2^{\text{BA}}) \quad (2.7)$$

Sarrafzadeh *et al.* (2005) has developed a diagnostics based on capacitance technology to measure on line the high motile cells and endospore concentration, without discriminate between them. As a result:

$$W_C = w_X^A + w_X^S \quad (2.8)$$

Where W_C is the specificity productivity of biomass for volume unit, except spores.

With reference to a CSTR the model reduces to the equation set reported in table 2.6 with the eq. (2.8): a set of 5 equations in 7 unknown (μ , μ_S , μ_D , X_A , X_S , X_D , Y_{ATP}). The solution of the model asks for the assessment of the relationships:

$$\mu = \mu(\text{AA, BA, B, Ac, Et, L, pH}) \quad (2.9)$$

$$Y_{\text{ATP}} = Y_{\text{ATP}}(\text{AA, BA, B, Ac, Et, L, pH}) \quad (2.10)$$

The theoretical approach highlights the relevance of acidogenic phase as information source for the process characterization. Typically, studies focused on solventogenic phase, neglecting the acidogenic phase since butanol is not produced.

2.1 Continuous fermentation with microfiltration unit to confine cells

The figure 2.1 refers to a CSTR equipped with a microfiltration unit. The volumetric flow rate Q is split in two streams: Q_1) the broth stream spilled from the

Table 2.6 Biomass balance in A.B.E fermentation

Total biomass	$W_X = w_X^A + w_X^S + w_X^D$	(T.2.6.1)
Acidogenic biomass	$w_X^A = \mu X_A - \mu_S X_A$	(T.2.6.2)
Solventogenic biomass	$w_X^S = \mu_S X_A - \mu_D X_S$	(T.2.6.3)
Eq. (2.7)	$X_A = \frac{Y_{\text{ATP}} (2f_1^{\text{AA}} + 3f_2^{\text{BA}})}{\mu}$	(T.2.6.4)

fermenter; Q_2) the sterile broth (permeate stream from the microfiltration unit). For the system of fig. 2.1 it results:

$$D = (d_1 + d_2) \quad (2.11)$$

$$d_1 = Q_1/V \quad (2.12)$$

$$d_2 = Q_2/V \quad (2.13)$$

$$W_X = d_1 X_T \quad (2.14)$$

The reactor system allows to tune the metabolites flow (associated to $Q = Q_1 + Q_2$) independently from the biomass flow (eq. 2.14).

The equation set of table 2.6 with eq.s (2.7) through (2.14) may be adopted to assess the variables under solventogenic conditions.

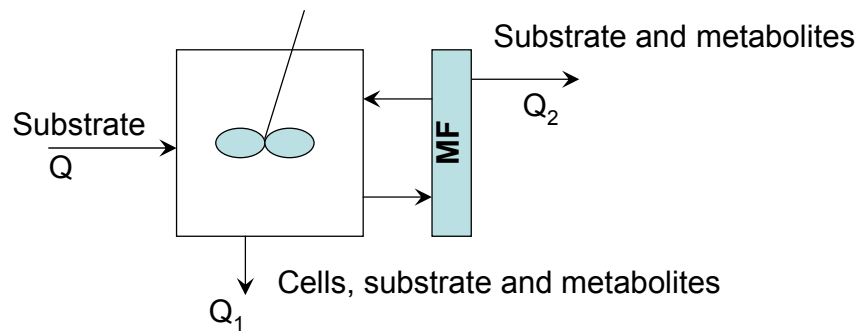


Figure 2.1. Sketch of a CSTR equipped with a microfiltration unit (MF).

3. BATCH CULTURES

This section reports the results obtained for batch cultures of *C. acetobutylicum*. Experiments were carried out to characterize the acidogenic and solventogenic phase in relationship with initial lactose concentration applying the principles typical of the chemical engineering. Tests were carried out adopting a lactose solution to mime whey permeate.

Batch tests carried out with whey permeate reported similar results in terms of yields, specific cells growth and solvent productivity, proving that the semi-synthetic medium adopted well mimics the wastewater streams of cheese industries.

3.1 An Assessment of the Kinetics of Butanol Production by *Clostridium acetobutylicum*

F. Napoli, G. Olivieri, A. Marzocchella, P. Salatino

School of Biotechnological Science

Dipartimento di Ingegneria Chimica-Università degli Studi di Napoli 'Federico II'

P.le V. Tecchio, 80 – 80125 Napoli, Italy

Results of a research activity aiming at investigating the feasibility of the acetone-butanol-ethanol (ABE) production by *Clostridium acetobutylicum* DSM 792 are reported. The kinetics of the ABE production process by free *C. acetobutylicum* in batch reactors has been assessed. A CSTR equipped with a microfiltration unit has been set-up and operated to assess the kinetics in the solventogenic phase. Solutions of lactose—concentration ranging between 10 and 100 g/L—were adopted as medium with the aim of emulating cheese whey. The conversion process was characterized in terms of cells, acids, solvents, pH and total organic compounds as a function of time. Tests carried out under batch conditions show that: i) cells growth is constant for lactose concentration (C_L) smaller than 100 g/L; ii) the butanol specific production rate - estimated at the onset of the solventogenic phase - as a function of C_L may be represented by a Monod-like kinetics; iii) the lactose conversion - measured at the end of the solventogenesis phase - decreases with C_L ; iv) the selectivity of butanol with respect to total solvents increases with C_L and stabilizes at about 72%_w for C_L larger than 30 g/L. Preliminary tests carried out under continuous conditions show that the process takes advantage from the steady state operation under solventogenic conditions. In particular, the butanol conversion rate is larger in the CSTR than in the STR, despite the lower final lactose concentration experienced in the CSTR.

KEYWORDS. Butanol, *Clostridium acetobutylicum*, kinetics, lactose, solventogenesis, batch, continuous

INTRODUCTION

Acetone-Butanol-Ethanol (ABE) fermentations have risen to a renewed interest as a way to upgrade biomass into valuable liquid fuels (Cascone, 2008). Recent developments of molecular techniques applied to solventogenic microorganisms in combination with advances in fermentation technology and downstream processing have contributed to improve ABE fermentation processes feasibility and competitiveness. The challenges raised over the last years as regards ABE production may be summarized into: i) use of renewable and waste-derived resources as substrates; ii) selection of strains characterized by high ABE productivity; iii) development of new fermentation systems; iv) development of new downstream strategies for enhanced solvent recovery.

The selection of unconventional substrates is favoured by the ability of *clostridia* strains to metabolize a wide range of carbohydrates – pentoses and hexoses - like glucose, lactose, etc., typically present in wastewater streams from, e.g., the food industry. In particular, studies available in the literature highlight the potential of ABE production by fermentation of lactose (Welsh and Veliky, 1984) or cheese whey (Maddox, 1980; Welsh and Veliky, 1986).

Even though *clostridia* have been proven successful to produce ABE, information available in literature to support industrial scale-up is still lacking (Villadsen, 2007; Cascone, 2008). The missing information regards either the kinetics of substrate conversion, cell growth and butanol production (Jones and Woods, 1986; Shinto et al., 2007) or the reactor systems. In a review on the state of art of the “white biotechnology”, Villadsen (2007) has specifically mentioned the ABE process as an example of how and how far a more fundamental based approach makes process development more effective and successful.

Results available in the open literature about ABE fermentation by *clostridia* strains regard mainly the metabolic pathway during fermentation of conventional substrates. The main steps of the fermentation process are: i) acidogenesis phase, characterized by cells growth coupled with the production of acids; ii) solventogenesis phase, characterized by growth stop, solvents production and acids conversion into solvents. Figure 1 reports a simplified pathway regarding the lactose conversion by *Clostridium acetobutylicum* (Bailey and Ollis, 1986; Jones and Woods, 1986). Among the key parameters, a relevant role plays the operating conditions that promote the switch between the acidogenesis phase, associated to the cells growth, and the solventogenesis phase, associated with the ABE production. A threshold value of the concentration of undissociated acids – acetyl and butyric – and of the pH have been identified as a trigger for the solventogenesis phase and growth stop. Another relevant aspect of the ABE production processes is related to the maximum butanol concentration that may be reached. In fact, *clostridium* activity is inhibited at butanol concentrations approaching about 20 g/L.

Typically, the ABE fermentation process has been studied by means of reactor systems belonging to the batch and fed-batch typologies. Only a few attempts are reported in literature regarding continuous fermentation by means of *clostridia* strains confined in the reactor by immobilization (Qureshi et al., 2000; Huang et al., 2004; Qureshi et al., 2006; Ezeji et al., 2007) or cell-recycling (Meyer and Papoutsakis, 1989; Tashiro et al., 2005). Some attempts are also reported in literature regarding two-stage conversions processes: the first stage devoted to produce acids, the second solvents (Ramey, 1998; Mutschlechner et al., 2000; Zhang et al., 2008).

The present study reports results of a research program aiming at investigating the feasibility of the ABE production by *Clostridium acetobutylicum* DSM 792 in a continuous biofilm reactor adopting cheese whey as feedstock. The specific contribution regards: i) the characterization of the kinetics of the ABE production process by free *C. acetobutylicum* under batch conditions; ii) the setting-up of a continuous reactor for the characterization of the kinetics of the conversion process under solventogenic conditions. Lactose solutions were adopted as medium with the aim of emulating cheese whey. The conversion process was characterized in terms of pH and concentration of cells, acids, solvents and total organic compounds. Results were worked out to assess the kinetics of the cells growth and of the ABE production. The yields of the carbon source in cells, acids and solvents were also assessed.

MATERIALS AND METHODS

Microorganism and culture media

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Stock cultures were

reactivated according to the DSMZ procedure.

Culture medium adopted consisted of Yeast Extract (YE) at 5 g/L and of D-Lactose at concentration ranging between 2 and 100 g/L. The medium was sterilized in autoclave.

Reactivated cultures were stored at 4°C in the stock medium made of 50 g/L D-Lactose culture medium supplemented with CaCO₃ (18 g/L).

Apparatus

Equipment for batch tests

Pre-cultures were carried out in 15 mL Hungate tubes. D-lactose bioconversion by *C. acetobutylicum* was investigated batchwise in screw-cap bottles (250 mL) housed in a thermostated room at 35°C.

Equipment for continuous tests

The apparatus for continuous bioconversion of lactose by free cells is represented in figure 2. It consisted of a CSTR equipped with a microfiltration unit and a process heater. Uniform agitation was achieved by a magnetic stirrer. The upper section of the reactor was equipped with ports for culture sampling, inoculation and feeding/purging streams. Isothermal conditions were maintained in the reactor by means of external heating and a jacket-exchanger enclosing the CSTR. The liquid stream was fed to the reactor by a peristaltic pump and was withdrawn as permeate stream from the microfiltration unit. A nitrogen stream was continuously metered with a rotameter at a constant flow rate and sparged at the vessel bottom to keep anaerobic conditions.

The overall volume of the bioreactor was 600 mL volume, about 5 mL as microfiltration cartridge and connection tubes. The cut-off of the microfiltration cartridge was 0.22 µm.

The reactor was operated continuously with respect to the gas and the liquid phases.

Operating conditions and procedures

All tests were carried out at 35°C under anaerobic conditions and no pH control was adopted.

Precoltures were prepared inoculating 2 mL of the stock culture into 5 mL of lactose supplemented synthetic medium and incubated for two days.

Batch tests

The precultures were inoculated into the reactors containing synthetic medium consisting of YE and lactose at pre-fixed concentration (up to 110 g/L). Typically, the initial cell concentration was fixed at 4 mg_{DM}/L. The culture was periodically sampled to measure cell and metabolites concentrations, until lactose concentration approached a stationary state. Each measurement was carried out in triplicate.

Concentration of substrate (C_L), cells (X) and metabolites (C_i) recorded during the tests were used to calculate the following data.

ξ_L overall lactose conversion, i.e. the ratio between the lactose converted and the initial lactose $(C_{L,0} - C_L)/C_{L,0}$

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

μ the specific growth rate of cells determined according to usual procedures for kinetic analysis from batch culture data under the assumption of first-order growth kinetics with respect to biomass concentration in the exponential phase. With the further hypotheses of small initial cell concentration and of large initial substrate concentration ($C_{L,0}$), μ was calculated as the slope of the $\ln(X/X_0)$ vs t plot in the exponential growth phase.

r_B^* the maximum butanol specific rate was estimated at the threshold of the solventogenesis

phase as the slope of the butanol concentration (C_B) vs time curve, divided for the cells concentration measured at the solventogenesis onset.

Continuous tests

The reactor volume (V_r) was set at 450 mL. 50 mL of preculture was inoculated in 400 mL medium with lactose concentration at the preset value. The fermentation was carried out under batch conditions until the onset of the solventogenesis phase. The lactose-bearing stream was fed at the rate (Q_0) set in accordance with the pre-set dilution rate ($D=Q_0/V_r$). At the end of the run the microfiltration cartridge was washed to remove entrapped/immobilized cells and the recovered dry biomass weighted. The culture was periodically sampled to measure cell and metabolites concentrations. Each measurement was carried out in triplicate. Data measured under steady state conditions were worked out to assess values of fractional yield coefficients and conversion degree, similarly to what reported for the batch tests.

Analytical methods

pH was measured off-line on 3 mL samples by a pHmeter (Hanna Instruments). Analysis of culture samples was carried out after centrifugation at 11,000 rpm for 10 min. The solid phase was characterized to determine biomass concentration. The liquid phase was characterized to determine lactose and metabolites concentrations and total organic carbon (TOC). 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 OD₆₀₀ corresponded to 0.4 g_{DM}/L. Elemental analysis of dry biomass was obtained by means of a C/H/N 2000 LECO® analyser. Lactose concentration was measured by means of an enzymatic kit (Biopharm). A GC apparatus was used, equipped with a FID, and outfitted with a capillary column poraplot Q (25 m x 0.32mm). External standards were adopted to assess acids and alcohols and their concentrations. The TOC was measured with a Shimadzu TOC 5000A analyzer.

THEORETICAL FRAMEWORK

The mass balance on carbon extended to the batch reactor volume takes into account the carbon content of the liquid phase and of *C. acetobutylicum* cells as well as that converted into CO₂, according to the Embden-Meyerhof pathway (Jones and Woods, 1986), during the observation time interval. A compact form of the global balance, referred to the acidogenesis phase [0, t_A], reads:

$$(TOC_0 + X_0\alpha_C) - (TOC_A + X_A\alpha_C) - 4 \frac{MW_C}{MW_L} (C_{L,0} - C_{L,A}) = 0 \quad (1)$$

where α_C is the carbon mass fraction of *C. acetobutylicum*, MW_C and MW_L the molecular masses of carbon and lactose, X and C_L the concentration of the cells and of lactose, respectively, and TOC the total organic carbon. Subscripts 0 and A refer to the beginning of the run and the time $t=t_A$, respectively. The accuracy of the carbon balance is expressed as:

$$\frac{(TOC_A + X_{max}\alpha_C) + 4 \frac{MW_C}{MW_L} (C_{L,0} - C_{L,A})}{(TOC_0 + X_0\alpha_C)} \approx \delta_{ac} \quad (2)$$

Extending the observation time interval to the whole test, extra CO₂ released during acetone production (Jones and Woods, 1986) must be taken into account. Assuming that the cell concentration decreases as a consequence of the sporulation without cell lysis, the global balance reads:

$$(TOC_0 + X_0\alpha_C) - (TOC_A + X_{max}\alpha_C) - 4 \frac{MW_C}{MW_L} (C_{L,0} - C_L) - \frac{MW_C}{MW_{Ac}} C_{Ac} = 0 \quad (3)$$

where X_{\max} is the maximum cell concentration and C_L and C_{Ac} the concentration of lactose and acetone at the end of the test. The accuracy of the test has been estimated by applying the eq. (2) to the whole test to compute δ_{ov} .

Under steady state conditions eq. (3) is modified as follows:

$$(TOC_0) - (TOC + X\alpha_c) - 4 \frac{MW_c}{MW_L} (C_{L,0} - C_L) - \frac{MW_c}{MW_{Ac}} C_{Ac} = 0 \quad (4)$$

where X , TOC , C_L and C_{Ac} are time-averaged values of concentrations. Subscript 0 refers to the value measured in the feeding. The accuracy of the material balances over the test has been expressed by δ_{ov} estimated as in the eq. (2).

The mass balance referred to each culture component extended to the continuous apparatus – CSTR and MF cartridge – under solventogenic conditions relies on the following assumptions:

- steady state operation;
- the feeding is aseptic and free of metabolites;
- cells are confined in the reactor;
- gas stripping of metabolites is neglected;
- reaction kinetics is represented by an unstructured model.

Accordingly, the following mass balance equations apply.

Lactose:

$$r_L = D \frac{C_{L,0} - C_L}{X} \quad (5)$$

where r_L is the specific conversion rate of the lactose.

Metabolites:

$$r_i = D \frac{C_i}{X} \quad (6)$$

where r_i is the specific production rate of the metabolite “i” and C_i the concentration of the metabolite “i” in the CSTR.

RESULTS

Batch tests

Figure 3 reports the time resolved profiles of the concentration of *C. acetobutylicum* cells (X), of lactose (C_L) and of metabolites (acetic acid, butyric acid, ethanol, acetone and butanol) as well as of pH, measured during a batch culture carried out at 50 g/L initial lactose concentration ($C_{L,0}$). It is possible to identify two phases: acidogenesis and solventogenesis. The onset of solvents production marks the beginning of the solventogenesis phase ($t_A \approx 22$ h). After a time lag lasting about 8 hours, the acidogenesis phase is characterized by: i) the steady increase of concentration of cells and acids; ii) a cell concentration vs time profile that mirrors lactose consumption; iii) the decrease of pH; iv) a molar ratio between acids (butyric/acetic) constant and equal to 1.5. The solventogenesis phase is triggered by the establishment of pH=4, in agreement with previous results (Jones and Woods, 1986). The solventogenesis phase is characterized by: i) the gradual decrease of the lactose concentration approaching a stationary value; ii) the acid molar ratio constant and equal to 1.5; iii) the gradual decrease of the cell concentration as a consequence of cell lysis (Mutschlechner et al., 2000; Ezeji et al., 2007).

The culture was further characterized in terms of: i) cells specific growth rate (μ); ii) fractional lactose-to-biomass yield estimated with reference to the acidogenesis phase ($(Y_{X/L})_{ac}$); iii) fractional lactose-to-butanol yield ($(Y_{B/L})_{solv}$) with reference to the solventogenesis phase; iv) fractional lactose-to-solvents yield ($(Y_{Sol/L})_{ov}$) and fractional lactose-to-butanol yield

($Y_{B/L}$)_{ov} with reference to the overall conversion; v) overall lactose conversion (ξ_L); vi) the maximum butanol specific rate (r_B^*) (see Fig. 2). With reference to the run reported in figure 3, the following values were obtained: $\mu=0.29\text{ h}^{-1}$; ($Y_{X/L}$)_{ac}=0.18; ($Y_{Sol/L}$)_{ov}=0.20; ($Y_{B/L}$)_{ov}=0.17; $\xi_L=0.34$; $r_B^*=80\text{ mg}_B/\text{g}_{DMh}$.

The elemental analysis of the dry biomass sampled at the end of the culture yielded $\alpha_C=40\text{ \%}_W$.

The accuracies of carbon balances for the test whose data are reported in figure 3 were $\delta_{ac}=0.96$ and $\delta_{ov}\square 0.90$.

Figure 4 reports experimental data collected during lactose conversion as a function of the initial lactose concentration. The accuracy of the tests expressed in terms of δ_{ac} and δ_{ov} was always between 0.90 and 1. The main results are hereinafter presented.

Acidogenesis phase (figure 4A). The concentrations of lactose, acetic and butyric acids measured at the end of the acidogenesis phase are reported. The process is characterized by cells specific growth rate, acid molar ratio and the ($Y_{X/L}$)_{ac} constant with $C_{L,0}$, under the operating conditions tested. Except for the run carried out at $C_{L,0}=2\text{ g/L}$, the value of pH at the threshold of the solventogenesis phase does not change with $C_{L,0}$. During the run carried out at $C_{L,0}=2\text{ g/L}$, pH decreases with time and stabilizes at 4.4 when the conversion stops as a consequence of lactose depletion. It is noteworthy that: i) the amount of lactose converted during the acidogenesis phase corresponds to a drop of lactose concentration (ΔC_L) of about 4 g/L, provided that the initial concentration is larger than this figure; ii) the combination of ($Y_{X/S}$)_{ac} and of the amount of lactose converted before t_A results in a cell concentration at the threshold of the solventogenesis phase nearly constant and equal to 0.7 g_{DM}/L; iii) cell growth is characterized by a zero-th order kinetics with respect to lactose with a specific growth rate of 0.29 h⁻¹ under the operating conditions tested.

Solventogenesis phase. Figure 4B reports data of butanol concentration and of lactose-to-butanol fractional yield estimated at the end of the solventogenesis phase. Analysis of the results highlights that: i) the final butanol concentration increases with $C_{L,0}$ and approaches a constant value at $C_{L,0}$ larger than 80 g/L; ii) the yield of lactose in butanol is characterized by a maximum at $C_{L,0}=50\text{ g/L}$.

Results regarding the overall conversion process carried out at $C_{L,0}$ ranging between 2 and 110 g/L (figure 4C) highlight some important features. The butanol fractional yield is characterized by a maximum of about 0.2 g_B/g_L at $C_{L,0}=50\text{ g/L}$, about half of the maximum theoretical value (0.4 g_B/g_L). The ξ_L and the overall selectivity with respect to butanol ($Y_{B/L}/Y_{Sol/L}$)_{ov} decrease with $C_{L,0}$ approaching, respectively, 0.3 and 0.65 (molar basis).

The maximum butanol specific production rate (r_B^*) is reported in figure 5 as a function of C_L . r_B^* may be described by a Monod-like model. Regression of data reported in figure 5 yields:

$$r_B^* = 0.13 \frac{C_L}{28 + C_L} \text{ (g}_B/\text{g}_{DMh}) \quad (7)$$

The plot of eq. (7) is also reported in figure 5.

Some information about the optimal operating conditions to use in batch bioconversion may be drawn by working out data reported in the previous figures. The yield to butanol with respect to the initial lactose concentration (product of lactose conversion and of $Y_{B/L}$) is characterized by a maximum of about 0.10 at $C_{L,0}=30\text{ g/L}$. Altogether, butanol productivity by fermentation increases with $C_{L,0}$, though its purity decreases as the initial lactose concentration increases. Therefore, operating conditions of a butanol production process should be optimized by taking into account the fermentation and the downstream recovery process at the same time. It should be underlined that in batch operation a fraction of

the available lactose is converted to stabilize an appropriate environment for butanol production (pH<4). In a continuous operation this fraction is consumed only during the reactor start-up.

Continuous tests

Figure 6 reports data measured during a test carried out in the CSTR equipped with the microfiltration unit, in order to confine cells within the reactor. The lactose concentration at the beginning of the batch culture and in the feeding was set at 50 g/L. The batch culture was stopped at the onset of the solventogenesis phase, i.e. as pH approached 4. The dilution rate of the reactor was set at 0.01 h⁻¹ until lactose depletion was detected, then at 0.04 h⁻¹. Steady state conditions established since t=240 h and lasted for four times the reactor space-time (=1/D). The time course of the concentration of cells, substrate and metabolites measured during the test conform to previous investigation carried out with other *clostridia* sp. and substrates under comparable operating conditions (Meyer and Papoutsakis, 1989; Tashiro et al., 2005). The steadiness of CSTR operation was verified by looking at the concentrations of substrate, cells and metabolites. The estimated value of δ_{av} (0.97) was very satisfactory.

The dried mass of cells recovered from the microfiltration cartridge was about 2.5% of the total amount of biomass present in the reactor. The possible contribution of this fraction to the reactor performance was therefore neglected.

Data measured under steady state conditions were worked out to obtain time-averages of the variables and conversion/production rates estimated according to eqs (5) and (6). Table 1 reports selected results referred to the run whose results are shown in figure 6.

The non-zero values of r_{AA} and r_{BA} reported in table 1 indicate that, in agreement with previous findings (Mutschlechner et al., 2000; Qureshi et al., 2000), even in the solventogenesis phase some cells are still able to produce acids and grow (Jones and Woods, 1986). The net specific growth rate of cells might be assessed by working out the production rate of both acids and the acid-to-solvent conversion rate. Future developments of the present set-up will be directed to enable the continuous monitoring of these variables.

When comparing results obtained in the continuously operated reactor with those of batch experiments, it can be noted that:

- the lactose conversion degree under continuous operation was higher than that measured under batch conditions, for the same initial lactose concentration;
- the specific production rate of butanol under continuous operation (table 1) was between two and three times the maximum production rate measured under batch conditions (figure 5), for the same lactose concentration (\cong 5 g/L);
- $Y_{B/L}$ and the selectivity to solvent were about the same for the batch and the continuous operation.

The improved performance of the microorganism measured under the continuous test

Table 1. Selected results corresponding to steady operation of the CSTR equipped with the microfiltration unit: $D=0.04$ h⁻¹; $C_{L,0}=50$ g/L

X , gDM/L	5.3	ξ_L , -	0.90
C_L , g/L	5.2	r_L , gL/h gDM	0.34
C_{AA} , g/L	0.52	r_{AA} , mg _{AA} /h gDM	3.9
C_{BA} , g/L	2.3	r_{BA} , mg _{BA} /h gDM	17
C_{Et} , g/L	0.54	r_{Et} , mg _{Et} /h gDM	4.1
C_{AC} , g/L	2.6	r_{AC} , mg _{Ac} /h gDM	20
C_B , g/L	9.9	r_B , mg _B /h gDM	75
$Y_{B/L}$, gB/gL	0.22	selectivity ($Y_{B/L}/Y_{Sol/L}$), molar basis	0.70

is in agreement with previous investigation carried out by Meyer and Papoutsakis (1989). The reduced demand of substrate for cell growth characteristic of the solventogenesis conditions enhances the specific butanol productivity.

The higher r_B measured in the continuous test highlights the effects of the conditions established in the reactor. The high value of pH and acids concentration promotes solvent production.

CONCLUSIONS

The conversion of lactose to Acetone-Butanol-Ethanol by *Clostridium acetobutylicum* has been studied with a focus on the assessment of the kinetics of acids and solvents production.

The specific growth rate of cells under acidogenesis conditions and the production rate of butanol at the onset of the solventogenesis phase have been characterized in batch experiments.

Conversion/production rates in the solventogenesis phase have also been investigated using a continuous reactor operated at steady-state and equipped with a microfiltration unit for confinement of microorganisms. The continuous fermentation process could be successfully operated for 13 days.

The comparison of the performances of the continuous versus batch processes confirms the great potential of the continuous process, which can be achieved without any prejudice for solvent selectivity and fractional yield. In fact, continuous operation turned out to yield larger lactose conversion and butanol production rates when compared with those achieved in batch operation, for comparable values of lactose concentration.

NOMENCLATURE

C	concentration, g/L
D	dilution rate, 1/h
Q	volumetric flow rate, L/h
r_i	conversion/production rate of the species "i", g/g _{DM} h
t	time, h
TOC	total organic carbon, g/L
V_r	reaction volume, L
X	cell concentration, g _{DM} /L
Y	fractional yield coefficient, g/g

Greek Letters

α_c	carbon fraction of cells, -
δ	error defined by eq. (2), -
ξ	conversion grade

Subscripts

0	initial/feeding value
ac	acidogenesis phase
A	onset of solventogenesis phase
AA, Ac, B, BA, Et, L	acetic acid, acetone, butanol, butyric acid, ethanol, lactose
C	carbon
ov	value of the variable referred to the overall batch test

REFERENCES

- Bailey, J.E., D.F. Ollis, *Biochemical Engineering Fundamentals*. McGraw-Hill, New York. 1986
- Cascone, R., "Biobutanol – A Replacement for Bioethanol?", *Chemical Engineering Progress*, 2008, Aug., S4-S9

- Ezeji, T.C., N. Qureshi, H.P. Blaschek, "Butanol Production from Agricultural Residues: Impact of Degradation Products on *Clostridium beijerinckii* Growth and Butanol Fermentation", *Biotechnology and Bioengineering*, 2007, vol. 97, 1460-1469
- Ezeji, T.C., N. Qureshi, H.P. Blaschek, "Bioproduction of Butanol from Biomass: from Genes to Bioreactors", *Current Opinion Biotechnology*, 2007, vol. 18, 220-227
- Huang, W.C., D.E. Ramey, S.T. Yang, "Continuous Production of Butanol by *Clostridium acetobutylicum* Immobilized in a Fibrous Bed Bioreactor", *Applied Biochemistry and Biotechnology*, 2004, vol. 113, 887-898
- Jones, D.T., D.R. Woods, "Acetone-Butanol Fermentation Revisited", *Microbiological Reviews*, 1986, Dec., 484-524
- Maddox, I.S., "Production of n-butanol from Whey Filtrate using *Clostridium acetobutylicum* N.C.I.B. 2951", *Biotechnology Letters*, 1980, vol. 2, 493-498.
- Meyer, C.L., E.T. Papoutsakis, "Continuous and Biomass Recycle Fermentations of *Clostridium acetobutylicum*. Part 1: ATP Supply and Demand Determines Product Selectivity", *Bioprocess Engineering*, 1989, vol. 4, 1-10.
- Mutschlechner, O., H. Swoboda, J.R. Gapes, "Continuous Two-Stage ABE-Fermentation using *Clostridium beijerinckii* NRRL B592 Operating with a Growth Rate in the First Stage Vessel Close to its Maximal Value", *J. Mol. Microbiology and Biotechnology*, 2000, vol. 2, 101-105.
- Qureshi, N., J. Schripsema, J. Lienhardt, H.P. Blaschek, "Continuous Solvent Production by *Clostridium beijerinckii* BA 101 Immobilised by Adsorption onto Brick", *World J. of Microbiology and Biotechnology*, 2000, vol. 16, 377-382.
- Qureshi, N., H.P. Blaschek, "Butanol Production from Agricultural Biomass", *Food Science and Technology*, 2006, vol. 148, 525-549.
- Ramey, D.E., "Continuous two Stages, Dual Path Anaerobic Fermentation of Butanol and other Organic Solvents using two Different Strains of Bacteria", 1998, Patent US 5753474.
- Shinto, H., Y. Tashiro, M. Yamashita, G. Kobayashi, T. Sekiguchi, T. Hanai, Y. Kuriya, M. Okamoto, K. Sonomoto, "Kinetic Modeling and Sensitivity Analysis of Acetone-Butanol-Ethanol Production", *Journal of Biotechnology*, 2007, vol. 131, 45-56
- Tashiro, Y., K. Takeda, G. Kobayashi, K. Sonomoto, "High Production of Acetone-Butanol-Ethanol with High Cell Density Culture by Cell-Recycling and Bleeding", *Journal of Biotechnology*, 2005, vol. 120, 197-206
- Villadsen, J., "Innovative Technology to meet the Demands of the White Biotechnology Revolution of Chemical Production", *Chemical Engineering Science*, 2007, vol. 62, 6957-6968
- Zhang, Y., W. Zheng, S. Wang, Y. Li, "Two-Stage Fermentation Process for Producing Butanol and Succinic Acid", 2008, Patent CN 101250561.
- Welsh, F.W., I.A. Veliky, "Production of Acetone-Butanol from Acid Whey", *Biotechnology Letters*, 1984, vol. 6, 61-64.
- Welsh, F.W., I.A. Veliky, "The metabolism of lactose by *Clostridium acetobutylicum*", *Biotechnology Letters*, 1986, vol. 8, 43-46.

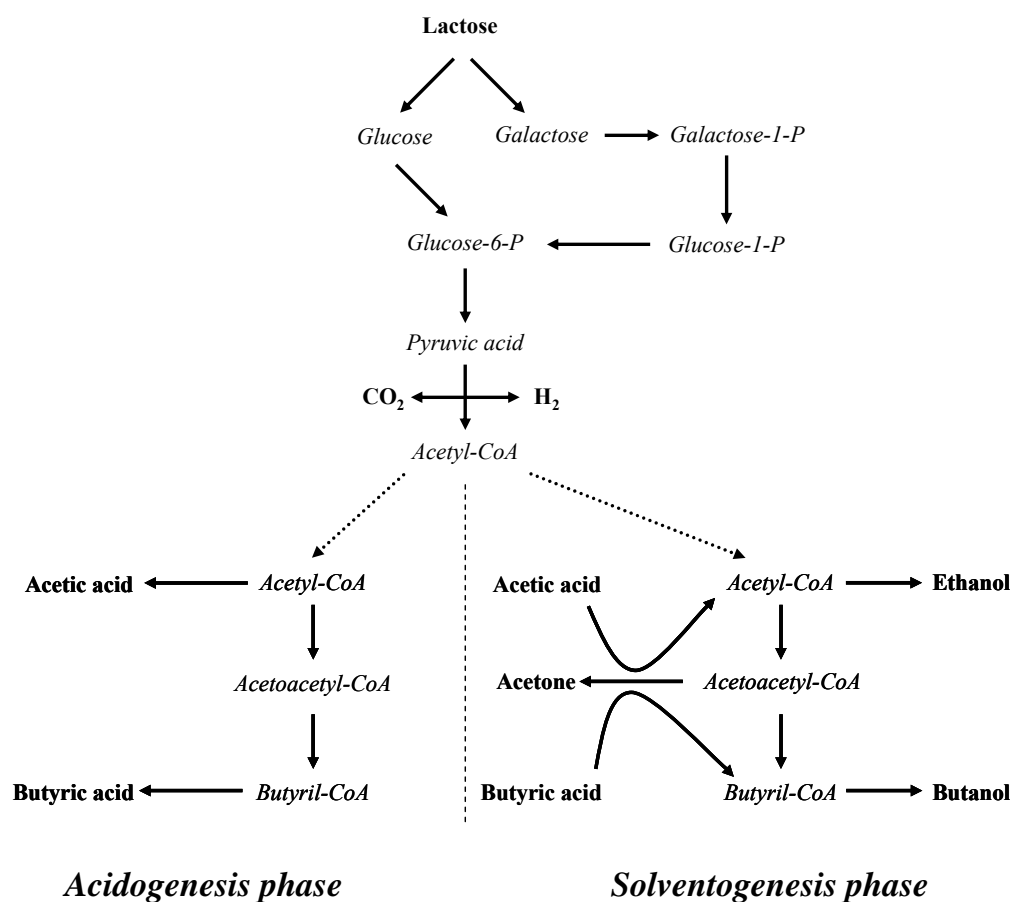


Figure 1. Simplified metabolic pathway of lactose conversion by *Clostridium acetobutylicum*. Slanted: intracellular metabolites. Bold: extracellular metabolites. (Bailey and Ollis, 1986; Jones and Woods, 1986).

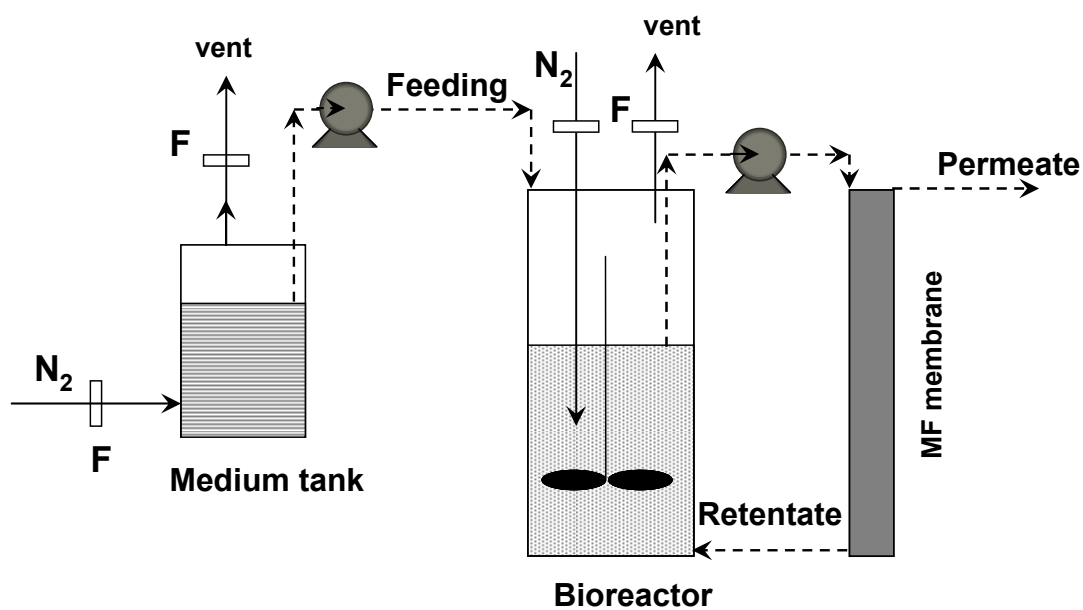


Figure 2. The apparatus used for continuous tests. F) gas sterilization filter.

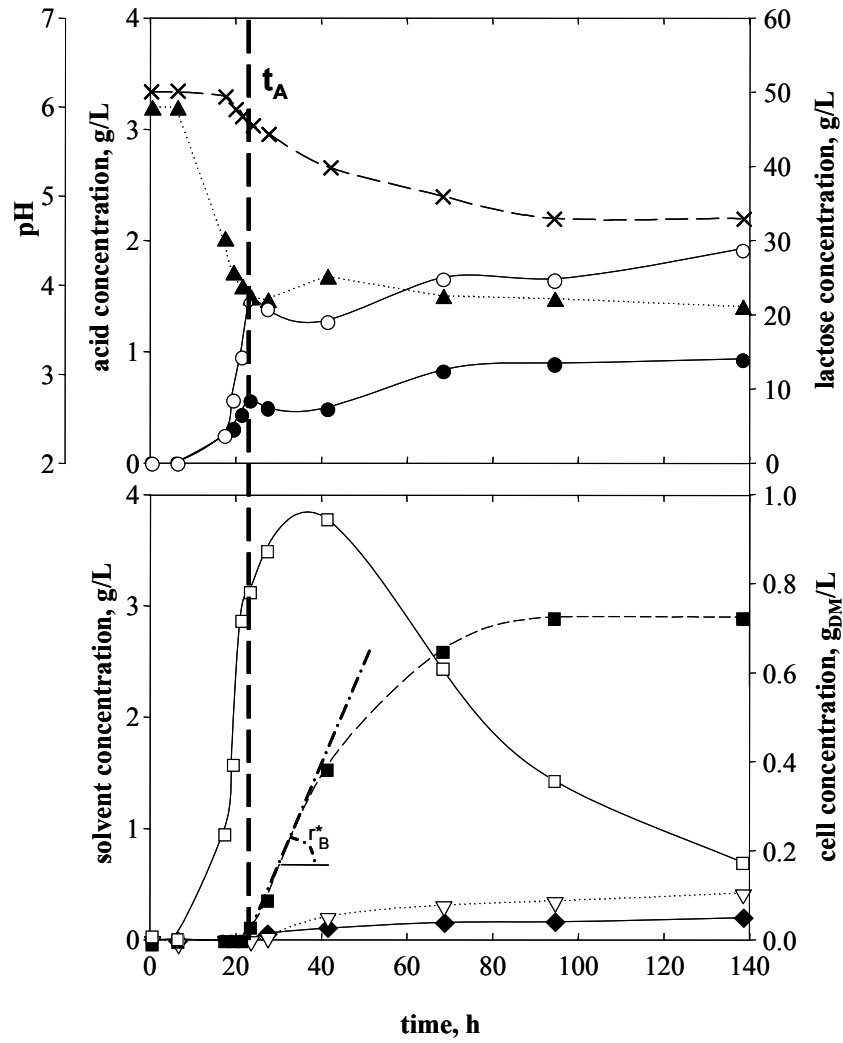


Figure 3. Data recorded during a batch test. $C_{L,0}=50$ g/L. The vertical dashed line marks the beginning of the solventogenesis phase. \blacktriangle pH, \bullet acetic acid, \circ butyric acid, \times lactose, \square cells, \blacklozenge ethanol, ∇ acetone, \blacksquare butanol.

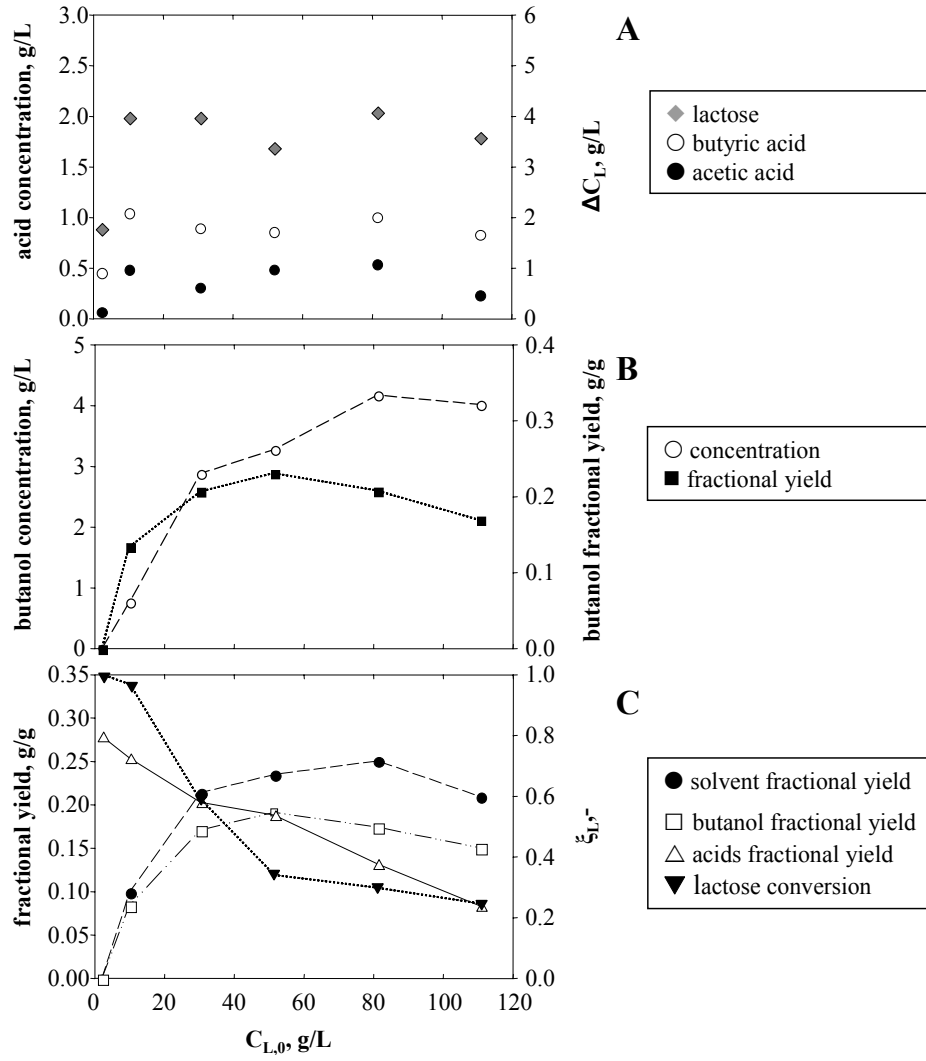


Figure 4. Data recorded during batch experiments. Lactose conversion as a function of the initial lactose concentration. A) Acidogenesis phase. B) Solventogenesis phase. C) Overall data.

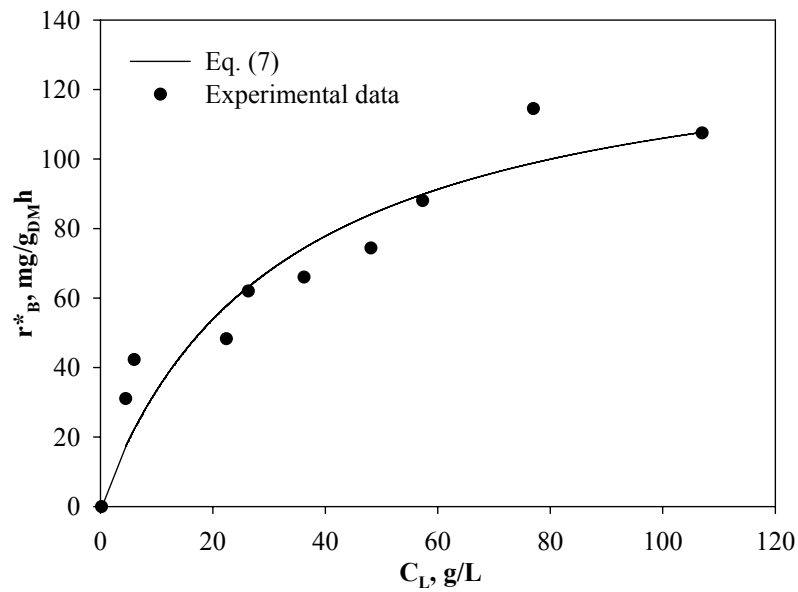


Figure 5. Maximum specific production rate of butanol r_B^* at the onset of the solventogenesis phase in batch cultures as a function of lactose concentration.

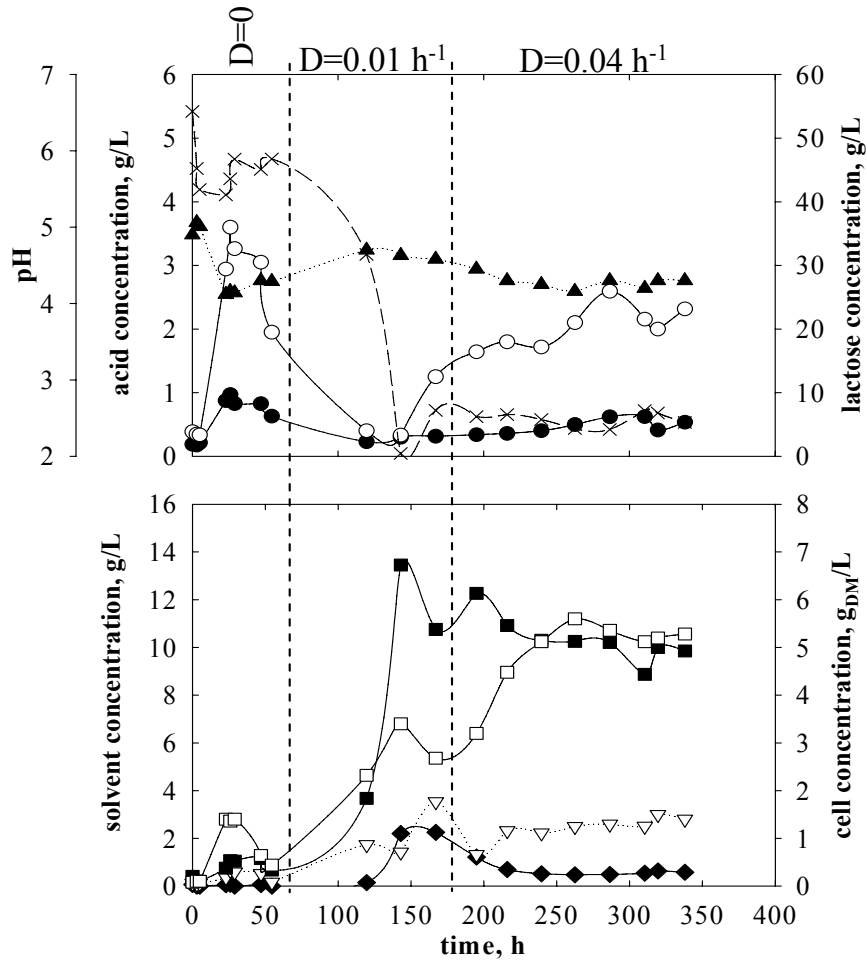


Figure 6. Data recorded during a continuous test carried out with the CSTR equipped with the microfiltration unit. $C_{L,0}=50$ g/L, $Y_E=5$ g/L. ▲ pH, ● acetic acid, ○ butyric acid, × lactose, □ cells, ◆ ethanol, ▽ acetone, ■ butanol.

4 CONTINUOUS CULTURES

This section reports on the characterization of the ABE fermentation process by adopting facilities operated under continuous conditions and characterized by uniform mixing. Data have been worked out in agreement with the typical methodologies of the chemical engineering to assess: the cells growth kinetics under acidogenic phase (§ 4.1), the cells and product yields under acidogenic conditions (§ 4.2), and the productivity under the solventogenic phase (§ 4.3).

4.1 Butanol Production by *Clostridium acetobutylicum* – I. Acidogenesis Kinetics

F. Napoli, G. Olivieri, M.E. Russo, A. Marzocchella, P. Salatino

*Dipartimento di Ingegneria Chimica - Università degli Studi di Napoli Federico II
P. V. Tecchio 80 - 80125 Napoli, Italy*

The growth kinetics of *Clostridium acetobutylicum* DSM 792 during acidogenesis phase under controlled conditions was investigated. Tests were carried out in a continuous stirred tank reactor equipped with a pH-controller. Synthetic medium was used supplemented with lactose as carbon source. Acids and solvents - typically self-produced - were eventually supplemented to investigate their inhibitory effect on the growth kinetics. The effect of pH was investigated in the range 4.0-7.0. The conversion process was characterized under steady state conditions at different values of dilution rate in terms of concentration of cells, acids, total organic carbon and pH. The growth kinetics of *C. acetobutylicum* DSMZ 792 on lactose was described adopting a multi-product inhibition model, including a novel formulation for the role of the pH. The 16 parameters of the model were evaluated. Particularly, the critical concentrations of products and the optimal and critical values of pH were assessed.

Keywords: ABE, *Clostridium acetobutylicum*, acidogenesis, product inhibition, growth model.

INTRODUCTION

The current scenario of energy sources has revived the interest for the production of solvents by fermentation of organic feedstocks. In this framework, production of butanol is gaining much interest because of its many advantages with respect other solvents in both biofuel and chemical feedstock markets (Cascone, 2008). The Acetone-Butanol-Ethanol (ABE) fermentation route still calls for investigation. New challenges regarding the ABE production include the use of renewable resources as substrates and the development of new and more efficient fermentation systems.

Though *clostridia* have been proven successful to produce ABE, information available in the literature to support industrial demonstration and scale-up is still lacking. Information on kinetics of substrate conversion, cell growth and butanol production is still extremely poor (Aiba and Shoda, 1969; Jones and Woods, 1986; Villadsen, 2007). Only few attempts are reported in the literature regarding continuous fermentation by means of *clostridia* strains confined in the reactor by immobilization or cell-recycling (Huang *et al.*, 2004; Qureshi and Blaschek, 2005; Ezeji *et al.*, 2007; Meyer and Papoutsakis, 1989; Tashiro *et al.*, 2005).

The metabolic process of *clostridia* strains is typically characterized by two phases (Jones and Woods, 1986): the acidogenesis phase during which substrates are converted in cells and acids (acetic and butyric acid); the solventogenesis phase during which substrates

and the produced acids are converted into solvents. The shift between the two phases strongly depends on pH and concentration of metabolites. The pH may influence both the maximum growth rate and the inhibitory potentials of metabolites. The hydrogenion concentration may affect the undissociated fraction of the organic acids that, in turn, is responsible for the acidogenesis/solventogenesis phases shift.

The inhibition effects of acids and solvents on the growth rate have been widely documented (Linden *et al.*, 1985; Jones and Woods, 1986; Yang and Tsao, 1994). Literature provides several models that describe cell growth taking into account the effect of few metabolites. Moreover, the experimental data were typically obtained under batchwise culture conditions (Gray and Wallis, 1983; Mulchandani and Volesky, 1986; Yerushalmi *et al.* 1986; Yang and Tsao, 1994).

Results regarding continuous fermentation (Meyer and Papoutsakis, 1989; Napoli *et al.*, 2009) point out that the acids are still produced during the solventogenesis phase. The contemporary presence of acidogenic and solventogenic cells makes the process modeling extremely complex. Acidogenic and solventogenic cells are morphologically different and they are characterized by different pathways (Long *et al.*, 1984; Jones and Woods, 1986).

The ability of *clostridia* strains to metabolize lactose has been highlighted in literature (Welsh and Veliky, 1984, 1986; Qureshi and Maddox, 1987). The lactose is the most abundant carbohydrate of the whey permeate and its removal is a critical issue for dairy industry. The typical features of the whey permeate - high BOD (110,000 ppm) and quite low sugar content (4-5%wt lactose) – do not fit for many fermentation processes, unless pre-concentration is adopted. Notwithstanding the mentioned characteristics, *clostridia* strains are able to convert whey permeate and successfully produce butanol.

The present study moves one further step toward the characterization of butanol production by *C. acetobutylicum*. This contribution regards modeling of the growth kinetics of suspended cells in a CSTR (Mutschlechner *et al.*, 2000). The results represent a relevant support for the modelling and the design of continuous reactors including biofilm reactors. The study addressed to the effects of acids (acetic and butyric), solvents (acetone, ethanol and butanol) and the pH on the growth rate of *C. acetobutylicum* under acidogenic state. Accordingly, the model proposes to describe acidogenic cells behaviour even under high ABE concentrations typical of solventogenic conditions, a mandatory step in the kinetics characterization of *C. acetobutylicum*.

MATERIALS AND PROCEDURE

2.1 Microorganism and culture media

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Stock cultures were reactivated according to the method suggested by the supplier. Culture medium (Napoli *et al.* 2009, Maddox and Ennis 1986) was supplied with 5 g/L Yeast Extract (YE) and D-Lactose as carbon source. The medium was sterilized in autoclave.

2.2 Apparatus

The adopted device was made by a magnetically stirred 1 Liter vessel (Pyrex®) equipped with temperature and pH controller. Temperature was controlled through a water jacket connected to a thermostatic water bath. Both inlet and outlet liquid streams were handled by peristaltic pumps (Gilson Minipuls 3). The volume of the liquid phase in the reactor (V) was set at 0.6 L.

The pH was adjusted at the desired value by a controller (Applikon Bio Controller ADI 1030) equipped with 1 M NaOH solution tank. Nitrogen was sparged at the bottom of the reactor to preserve the anaerobic condition.

2.3 Analytical methods

Analysis of culture samples withdrawn from the chemostat provided biomass concentration, lactose and metabolites concentration and total organic carbon (TOC) in the liquid phase. Cell density was measured as optical absorbance at 600 nm (OD_{600}) using a spectrophotometer (Cary-Varian mod. 50 scan UV-VIS spectrophotometer). Calibration tests for *C. acetobutylicum* dried mass indicated that $1 OD_{600} = 0.4 g_{DM}/L$. Elemental analysis of dry biomass was performed by means of a C/H/N 2000 LECO® analyser. Lactose concentration was measured by means of enzymatic kit (Biopharm). Metabolites concentrations were measured by means of a gas-chromatograph (Agilent 7890A GC) equipped with a FID and with a capillary column poraplot Q (25 m x 0.32mm), adopting external standards. The TOC was measured by a Shimadzu TOC V-CSH analyser.

2.4 Operating conditions and procedures

Precultures were prepared inoculating 2 mL of the reactivated cells into 50 mL of lactose supplemented semi-synthetic medium and incubated for two days.

The precultures were inoculated into the reactor containing 0.6 L semi-synthetic medium consisting of YE and lactose at fixed concentration.

Typically, after 6 h of batch culture the lactose bearing stream was fed to the reactor at the desired dilution rate (D). The culture was periodically sampled to measure cell and metabolites concentrations, until lactose concentration approached a stationary state. Steady states were characterized in terms of average concentration of lactose, cells and metabolites measured over a time period larger than 5-6 times the average liquid residence time.

All tests were carried out at 35°C under anaerobic conditions. The lactose concentration in the inlet stream was set between 4 and 50 g/L. The set-point for the pH controller was fixed at values ranging between 4.0 and 7.0. The dilution rate was changed between 0.05 and 0.77 h⁻¹.

The reactor vessel and the medium were sterilized in autoclave. The gas stream was sterilized by filtration (cut-off 0.2 µm, Millipore).

3. THEORETICAL FRAMEWORK

The reliability of the data measured during the tests was checked by means of the mass balance on carbon. The assumptions adopted to develop the carbon balance were: i) the chemostat was at steady state regime; ii) the only acidogenesis pathway was active; iii) carbon conversion to CO₂ followed the Embden-Meyerhof pathway (Jones and Woods, 1986).

$$(TOC_0 + X_0 \cdot \alpha_c) - (TOC + X \cdot \alpha_c) - 4 \frac{MW_C}{MW_L} (L_0 - L) = 0 \quad (1)$$

where α_c is the carbon mass fraction of *C. acetobutylicum* cells (MW_C and MW_L the molecular weight of carbon and lactose, X , L and TOC the concentration in the liquid phase of cells, lactose and total organic carbon respectively (subscripts “0” refers to the reactor inlet stream). The accuracy of the measurements has been expressed by δ defined as:

$$\frac{(TOC_0 + X_0 \cdot \alpha_c) - (TOC + X \cdot \alpha_c) - 4 \frac{MW_C}{MW_L} (L_0 - L)}{(TOC_0 + X_0 \cdot \alpha_c)} = \delta \quad (2)$$

Results of the continuous tests were worked out in agreement with the material balance referred to cells extended to the continuous reactor and relying on the following assumptions:

- the feeding is aseptic and free of metabolites;
- gas stripping of metabolites is negligible with respect to their production rate;
- reaction kinetics is represented by an unstructured model;

$$\frac{dX}{dt} = (\mu - D)X \quad (3)$$

where X is the cell concentration, μ is the specific growth rate and D is the dilution rate.

Under steady state conditions eq. (3) reads:

$$D = \mu \quad (4)$$

The growth kinetics of *C. acetobutylicum* is characterized by products inhibition and typically the specific growth rate drops to zero when the concentration of products approaches a critical value. With reference to a single-product inhibited growth, the models available in literature may be traced back to three classes (Han & Levenspiel, 1988):

the Ierusalimsky-type

$$\mu = \mu_{\max} \frac{S}{S + K_S} \frac{1}{1 + P/K_P} \quad (5)$$

the Aiba-type

$$\mu = \mu_{\max} \frac{S}{S + K_S} e^{\left(-\frac{P}{K_P}\right)} \quad (6)$$

the Luong-type

$$\mu = \mu_{\max} \frac{S}{S + K_S} \left(1 - \frac{P}{P_{\max}}\right)^n \quad (7)$$

where S and P are the substrate and the product concentrations and K_S , K_P and n model parameters. However, both models (5) and (6) cannot predict zero growth rate for a finite inhibitor concentration. On the contrary, model (7) was proposed having such property that is mostly connected to toxic product. An extension of the Luong model has been adopted in the present study for the growth kinetics of *C. acetobutylicum* under acidogenesis conditions in agreement with Zeng *et al.* (1991 and 1994). Considering the main products of the *C. acetobutylicum* lactose fermentation the multiproduct-inhibited growth model at a fixed value of pH reads:

$$\mu = \mu_{\max} \cdot \frac{L}{L + K_L} \cdot \left(1 - \frac{AA}{AA_{\max}}\right)^{n_{AA}} \cdot \left(1 - \frac{BA}{BA_{\max}}\right)^{n_{BA}} \cdot \left(1 - \frac{Ac}{Ac_{\max}}\right)^{n_{Ac}} \cdot \left(1 - \frac{Et}{Et_{\max}}\right)^{n_{Et}} \cdot \left(1 - \frac{B}{B_{\max}}\right)^{n_B} \quad (8)$$

where L , AA , BA , Ac , Et and B are the concentration of lactose, acetic acid, butyric acid, acetone, ethanol and butanol respectively, μ_{\max} the maximum specific growth rate, K_L the Monod coefficient for lactose. The inhibitory effect of solvent is described in eq. (8), even though these metabolites are not present during acidogenesis phase. The interest in these effects is fueled by the necessity to describe the kinetics of acidogenic cells even during solvent production.

The assessment of the 12 parameters μ_{\max} , K_L , AA_{\max} , BA_{\max} , Ac_{\max} , Et_{\max} , B_{\max} , n_{AA} , n_{BA} , n_{Ac} , n_{Et} and n_B has been carried out adopting a successive approximation strategy. In particular, the operating conditions adopted for the tests were selected in order to force the chemostat towards peculiar steady states. The main steps of the strategy are reported hereafter:

- the assessment of $\mu_{\max}|_{pH^*}$ and K_L by working out data measured during tests carried out at pH=5 and at D close to the wash-out conditions. Tests were characterized by low lactose conversion, than low metabolites concentration;
- the assessment of the critical concentration $P_{x,\max}$ of each metabolite (AA_{\max} , BA_{\max} , Ac_{\max} , Et_{\max} , B_{\max}) and of each exponents n_{AA} , n_{BA} , n_{Ac} , n_{Et} and n_B by working out data

measured during tests carried out at pH=5 and adding the metabolite “P_x” investigated to the reactor feed.

The effects of pH on the maximum specific growth rate and on the critical concentrations of metabolites were assessed working out data measured during tests carried out at fixed pH ranging between 4 and 7.

RESULTS

Table 1 reports a representative set of runs carried out at pH = 5.0, dilution rate ranged between 0.22 and 0.72 h⁻¹. The elemental analysis of the dry biomass sampled at the end of each run yielded: α_C=44 %_w. The table reports the accuracy (δ) of the measured carbon consumption at the steady state assessed in agreement with eq. (2), it resulted smaller than 10 %. Whatever the operating conditions adopted the metabolites concentrations were quite low.

Data reported in table 1 - and during tests characterized by low metabolites concentrations - were worked out to assess a rough value of μ_{max} and K_L at pH=5. In particular, neglecting the inhibition terms in eq. (8) data regression yielded μ_{max}^I=0.80 h⁻¹ and K_L=2.0 g/L, where the prime marks the value as a first assessment.

Acids inhibition

Table 2 reports data regarding tests carried out in order to assess the critical concentration AA_{max} and AB_{max} and the exponents n_{AA} and n_{AB}. The first four rows refer to tests characterized by low lactose conversion and the medium supplemented with either acetic or butyric acid up to high inhibiting concentrations. Under these operating conditions the produced acids may be neglected with respect to the supplemented one and eq. (8) approaches the following forms depending on the added acid:

$$\mu = \mu_{\max}^I \cdot \frac{L}{L + K_L^I} \cdot \left(1 - \frac{AA}{AA_{\max}^I}\right)^{n_{AA}} \quad (9)$$

$$\mu = \mu_{\max}^I \cdot \frac{L}{L + K_L^I} \cdot \left(1 - \frac{BA}{BA_{\max}^I}\right)^{n_{BA}} \quad (10)$$

Accordingly, the inhibition effect of the acid “i” may be assessed by analyzing the ratio:

$$R_i = \frac{\mu}{\mu_{\max}^I \frac{L}{L + K_L^I}} = \left(1 - \frac{P_i}{P_{\max}}\right) \quad (11)$$

as a function of the concentration of the acid itself. Figure 1 shows data of R_i assessed with reference to the results reported in the first four rows of table 2. Assuming the exponent n_i=1, the extrapolation of data in figure 1 at R_i=0 yields the first approximation of the critical acids concentrations AA_{max}^I=1.66 g/L and BA_{max}^I=3.34 g/L.

The procedure for the assessment of the exact value of the parameters in eq. (8) (μ_{max}, K_L, AA_{max}, BA_{max}) evaluated at pH=5.0 was based on the processing of data in the tables (1) and (2) that concern wide intervals of dilution rate, lactose conversion and metabolites concentration. The difference between the measured specific growth rate □ and that estimated by means of eq.(8) was minimized by means of statistical functions of the Microsoft Excel software. The approximated values assessed for the parameters of eq. (8) were adopted as starting point of the procedure. The resulting values of the model parameters were:

$$\begin{aligned} \mu_{\max} &= 0.95 \pm 0.07 \text{ h}^{-1} & K_L &= 1.34 \pm 0.06 \text{ g/L} \\ AA_{\max} &= 1560 \pm 60 \text{ mg/L} & n_{AA} &= 0.98 \pm 0.07 \\ BA_{\max} &= 3000 \pm 180 \text{ mg/L} & n_{BA} &= 0.96 \pm 0.15 \end{aligned} \quad (12)$$

The critical concentration of butyric acid is larger than that of the acetic acid in agreement with the higher selectivity towards the butyric acid assessed during the runs carried out without any supplemented acid. So, the acetic acid concentration in the chemostat is always lower than the butyric acid concentration even if acetic acid formation from lactose is favored in terms of energy balance.

Solvent inhibition

The inhibition effects of solvents on the growth rate under acidogenic conditions have been investigated supplementing acetone, butanol and ethanol to the reference medium. In particular the lactose concentration in the feed medium was set at a concentration about ten fold larger than K_L . Relevant data of the tests carried out supplementing a single solvent to the medium are reported in table 3. The inhibition ratio R_i was defined as the ratio between the measured growth rate μ and the growth rate calculated adopting the assessed parameter:

$$R_i = \frac{\mu}{\mu_{\max} \left(1 - \frac{AA}{AA_{\max}}\right)^{n_{AA}} \left(1 - \frac{BA}{BA_{\max}}\right)^{n_{BA}}} \quad (13)$$

Figure 2 shows the inhibition ratio calculated for tests carried out supplementing butanol to the medium as a function of the solvent concentration. The shadow area marks the interval of investigated concentration that give rise to complete inhibition leading the reactor toward wash-out even when dilution rate was fixed at very small values ($0.05-0.1h^{-1}$). This finding suggests that a non-linear function characterizes the dependence of μ on the butanol concentration.

The values of B_{\max} and n_B , assessed by means eq. (8), are 16500 mg/L and 0.47 respectively, with an error of 4%. The agreement between the R_B measured and the value assessed by eq. (13) (fig. 2) is satisfactory. The critical value assessed for the butanol concentration is in agreement with experimental data and with the value reported in literature (Qureshi *et al.*, 1988; Yang and Tsao, 1994).

Figure 3 shows the inhibition ratio estimated for both ethanol and acetone in agreement with eq. (13). It should be noted that the maximum concentration of acetone and ethanol investigated were quite larger than that usually achieved during the ABE fermentation. Accordingly and assuming that the exponent for both solvent is 1, the critical concentration can be assessed by linear extrapolation of data at $R_i=0$. It resulted $Ac_{\max}=69.0\pm1.4$ g/L and $Et_{\max}=38.5\pm3.8$ g/L. The agreement between the R_i measured and the value assessed by eq. (13) (fig. 3) is satisfactory for both solvent.

The Effect of pH

The role of the pH on the acidogenesis kinetics can be twofold. The hydrogenionic concentration could affect both the maximum specific growth rate and the inhibitory terms that depend on the concentration of undissociated acids.

Table 4 reports steady state data of continuous cultures carried out at pH values ranging between 4.0 and 7.0. Data regarding the tests carried out at pH=5.0 are reported in table 1 and table 2 (no acids-supplemented medium). The data at each pH were worked out in agreement with eq. (8) taking into account the acid either as undissociated or as total. The regression of data adopting the undissociated acids concentrations was unsuccessful. On the contrary, the data regression adopting total acids concentration was successfully and the analysis has pointed out that: i) the pH affects the specific growth rate; ii) the critical concentration of acids (AA_{\max} , AB_{\max}) does not change with pH.

The data of the specific growth rate vs the pH reported in Fig. 4 were analyzed in agreement with the model available in literature proposed by Bailey and Ollis (1986) and by Tang *et al.* (1989):

$$\mu_{\max} = \frac{\bar{\mu}_{\max}}{1 + \frac{H^+}{K_H} + \frac{K_{OH}}{H^+}} \quad (14)$$

Where H^+ is the concentration of hydrogen ions, $\bar{\mu}_{\max}$, K_H and K_{OH} model parameters. Noteworthy, the pH characterized by the maximum cell growth rate (pH_{OPT}) is related to the parameters K_H and K_{OH} :

$$-\log(\sqrt{K_H K_{OH}}) = pH_{OPT} \quad (15)$$

The regression of data in Fig. 4 in agreement with eq. (14) yields:

$$K_H = 3.33 \cdot 10^{-4} \text{ M} \quad K_{OH} = 5.04 \cdot 10^{-7} \text{ M} \quad \bar{\mu}_{\max} = 1.06 \text{ h}^{-1} \quad pH_{OPT} = 4.9$$

The comparison between the data reported in figure 4 and the plot of eq. (14) shows that the agreement is satisfactory for pH larger than 5.0, poor for pH smaller. The typical symmetry of the eq. (14) does not reproduce the experimental data characterized by a steep decrease of specific growth rate at pH lower than 5.0.

The steep decrease of μ_{\max} at pH lower than 5.0 may be analyzed taking into account the observation reported Huang *et al.* (1985) as regards the growth of *C. acetobutylicum* ATCC 824 on glucose. The Authors reported that the difference of pH between the liquid bulk and the intracellular value is: i) close to zero for pH larger than the optimal value for the cell growth rate (pH_{OPT}); ii) as large as 1.5 for pH smaller than pH_{OPT} . Accordingly, a new model for the μ_{\max} vs pH has been proposed:

$$\mu_{\max} = \frac{\bar{\mu}_{\max}}{1 + \frac{H^+}{K_H} + \frac{K_{OH}}{H^+}} \left(1 - \frac{10^{(pH_{OPT} - pH)}}{10^{\Delta pH}} \right) \quad (16)$$

The plot of eq. (16) has been reported in Fig. 4 (continuous line) assuming: $pH_{OPT} = 4.9$ and $\Delta pH = 1.5$. The agreement between experimental data and the proposed model (eq. 16) is satisfactory over the entire range of the investigate pH values.

Figure 5 reports the comparison between the experimental values of specific growth rate and the theoretical values calculated in agreement with eq.s (8) and (16) adopting the parameters reported in table 5. Data refers to the tests carried out at pH larger than 4.2. The error was lower than 10% for almost all the tests suggesting that the proposed model (eq.s 8-16) satisfactorily describes the growth kinetics of *C. acetobutylicum* DSMZ 792 on lactose supplemented medium.

DISCUSSION

The results presented in the previous sections deserve further discussion. The attention has been focused on three issues: i) the critical concentration of acids; ii) the critical concentration of the solvents; iii) the range of pH characteristics of the solventogenesis phase.

The critical concentration assessed for butyric acid resulted about twice (3.00 g/L) than that of the acetic acid (1.56 g/L), whatever the operating conditions investigated. Accordingly, the production of butyric acid resulted larger notwithstanding the acetic acid formation from lactose is energetically favored.

An interesting scenario results from the comparison of the critical concentration assessed in the present study and data reported in literature. Yang and Tsao (1994) assessed a toxic concentration of acetic acid and butyric acid of about 12 and 11 g/L, respectively, for batch culture of *C. acetobutylicum* ATCC824 on complex medium supplemented with glucose. The industrial strain *C. acetobutylicum* P262 was characterized by critical concentration of acetic and butyric acids of 4.8 and 10.6 g/L, respectively, for batch

fermentation of medium close to that adopted in the present study (Ennis and Maddox, 1986). The analysis of the critical concentration suggests that the toxic concentration of acids depends on the adopted strain as well as on the medium composition.

The comparison among the critical concentration of the acids and the solvents points out that the most oxidized form of metabolites are more toxic than the alcohol state, the critical concentration of the latter being an order of magnitude larger. The difference is in agreement with the different pathway responsible of the inhibition phenomenon. Moreover, the larger value of the critical concentration of the solvents is in agreement with the acidogenesis-solventogenesis shift. In fact, as the environment become toxic for the microorganism during the acidogenesis phase its metabolism shifts in the production of less toxic species (solventogenic phase).

The analysis of the critical concentration of the three solvents suggests that the inhibitory effect of acetone and ethanol during the fermentation is negligible with respect of that of butanol. Therefore, strategies aiming at the optimization of the process may be developed confining the attention only on the inhibition effect of the butanol.

The pH appears to play a twofold effect on *C. acetobutylicum* cell growth. The first effect is common for most of the bacteria strains and is related to the active fraction of enzymes as detailed by Tang et al. (1989). However, the model proposed by Tang et al. (1989) appears to be successful for pH larger than the value corresponding to the optimum growth rate. The second effect is related to the ability of the cells to survive under un-optimal environment: as the pH in the medium drops below a threshold value the cells pumps hydrogenion out keeping the intracellular pH close to the optimum. Accordingly, the pH intervals of optimal cell growth and of solvent production are different and typically change with the strain. A number of the *C. acetobutylicum* DSM strains produce solvents at pH well smaller than the optimum value for the growth, about 5.0 (Bahl et al., 1982; Nishio et al., 1983). Typically, the optimal pH to produce solvents is 4.3, even if production is still active at pH as low as 3.8. *C. acetobutylicum* ATCC 824 grows at pH=6 and produce solvents at pH between 5.5 and 4.3 (Monot et al., 1984). Kim et al. (1982) reported that the production of solvents was practically absent at pH=5.8 and was very satisfactory at pH=4.5. The optimum pH range for solvent production is much higher for *C. acetobutylicum* P262 and related strains (P265,P270), which were used for the industrial production of solvents (Ennis and Maddox, 1986). The analysis of these results suggests that a ΔpH between - maximum cell growth rate conditions and optimal solvent production - of about 1.5 is a sound proposal for the model eq. (16).

CONCLUSION

The growth kinetics of *Clostridium acetobutylicum* DSM 792 using lactose as substrate was successfully investigated by means of a uniformly mixed reactor operated under continuous conditions. The chemostat was operated under pH controlled conditions to investigate the acidogenic state of the microorganism. The pH ranged between 4.0 and 7.0. Acetic and butyric acids and ethanol, acetone and butanol as well - solvents typically produced by the investigated microorganism under solventogenic phase - were supplemented to the medium to characterize their effects on the kinetics.

A multiproduct-inhibited growth model was proposed in agreement with Zeng et al. (1991 and 1994) and Tang et al. (1989). In particular, a new relationship was proposed to describe the effect of pH on the specific growth rate of *C. acetobutylicum* DSMZ 792. The 16 model parameters were successfully assessed by adopting a successive approximation strategy. The model proposes as a tool to characterize the microorganism under a wide interval of operating conditions, even for acidogenic cells under solventogenesis phase.

Acknowledgments

Dr. Fabio Napoli thanks prof. A.P. Zeng of the TU-Hamburg for the fruitful discussions. The Authors are also indebted with Mrs Maria Elena Compare and Mrs Ottavia Pangia for their assistance in experimental investigation.

NOMENCLATURE

AA, Ac, B, BA, Et, L concentration of acetic acid, acetone, butanol, butyric acid, ethanol, (g/L); lactose (mg/L)
D dilution rate, 1/h
 H^+ hydrogenion/hydroxylic concentration, M
 R_i inhibition ratio referred to the species “i”, -
TOC total organic carbon, g/L
X cells concentration, g_{DM}/L

Greek Letters

α_c carbon fraction of cells, g/g_{DM}
 δ error defined by eq. (2),
 μ specific growth rate, h⁻¹

Subscripts

0 feed medium
AA, Ac, B, BA, Et, L acetic acid, acetone, butanol, butyric acid, ethanol, lactose

REFERENCES

- Aiba, S. and M. Shoda, “Reassessment of product inhibition in alcohol fermentation” J. Ferment. Technol., 1969, vol. 47, 790-794
- Bahl, H., W. Andersch, K. Braun, and G. Gottschalk, 1982. “Effect of pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* grown in continuous culture”, Eur. J. Appl. Microbiol. Biotechnol., 1982, vol.14, 17-20
- Bailey, J.E. and Ollis D.F. (1986), “Biochemical engineering fundamentals” Second Edition. McGraw-Hill Inc., New York.
- Cascone, R., “Biobutanol - A replacement for bioethanol?”, Chem. Eng. Prog., 2008, August, S4-S9
- Ezeji, T.C., N. Qureshi and H.P. Blaschek, “Bioproduction of butanol from biomass: from genes to bioreactors”, Curr. Op. Biotechnol., 2007, vol. 18, 220-227
- Ennis, B.M. and I.S. Maddox, The effect of pH and Lactose concentration on solvent production from whey permeate using *C.acetobutylicum*, 1986, Biotechnol. Bioeng, 1987, vol. 29, 329-334.
- Gray, G. C. and Wallis, D. A., “Analysis of the acetone butanol fermentation relative to bioreactor characteristics”, Biotechnol. Bioeng. 1983, vol. 13, 371-376
- Han, K., Levenspiel, O. (1988), Extended Monod-equation for substrate, product and cell inhibition, Biotech. Bioeng. 32, 430.
- Huang, W.C., D.E. Ramey and S.T. Yang, “Continuous production of butanol by *Clostridium acetobutylicum* immobilized in a fibrous bed”, Bioreactor Appl. Biochem Biotechnol, 2004, vol. 113, 887-898
- Jones, D. T., A. Van der Westhuizen, S. Long, E. RI. Allcock, S. J. Reid, and D. R. Woods, “Solvent production and morphological changes in *Clostridium acetobutylicum*”. Appl. Environ. Microbiol., 1982, vol. 43, 1434-1439.
- Jones, D.T. and D.R. Woods, “Acetone-butanol fermentation revisited” Microb. Rev., 1986, Dec, 484-524

- Kim, B. H., P. Bellows, R. Datta, and J. G. Zeikus, "Control of carbon and electron flow in *Clostridium acetobutylicum* fermentations: utilization of carbon monoxide to inhibit hydrogen production and to enhance butanol yields" *Appl. Environ. Microbiol.*, 1984, vol 48, 764-770
- Huang, L., L. N. Gibbins, C. W. Forsberg, Transmembrane pH gradient and membrane potential in *Clostridium acetobutylicum* during growth under acetogenic and solventogenic conditions, *Applied and Environmental Microbiology*, 1985, vol. 50, 1043-1047
- Linden, J.C., A.R. Moreira and T.G. Lenz, – "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.
- Long, S., D.T. Jones and D.R. Woods, "Initiation of solvent production, clostridial stage and endospore formation in *Clostridium acetobutylicum* P262." *Appl. Microbiol. Biotechnol.*, 1984, vol. 20, 256-261.
- Luong, J. H. T., "Kinetics of ethanol inhibition in alcohol fermentation", *Biotechnol. Bioeng.*, 1985, vol. 27, 280-285.
- Meyer, C.L. and E.T. Papoutsakis, "Continuous and biomass recycle fermentations of *Clostridium acetobutylicum*. Part 1: ATP supply and demand determines product selectivity", *Bioprocess Engineering*, 1989, vol. 4, 1-10
- Monot, F., J. M. Engasser, and H. Petitdemange, "Influence of pH and undissociated butyric acid on the production of acetone and butanol in batch cultures of *Clostridium acetobutylicum*" *Appl. Microbiol. Biotechnol.*, 1984, vol. 19, 422-426.
- Mulchandani, A. and B. Volesky, "Modelling of the acetone-butanol fermentation with cell retention" *The Canadian Journal of Chemical Engineering*, 1986, vol. 64, 625-631
- Mutschlechner, O., H. Swoboda and J.R. Gapes, "Continuous two-stage ABE-fermentation using *Clostridium beijerinckii* NRRL B592 operating with a growth rate in the first stage vessel close to its maximal value" *J. Molecular Microbiology and Biotechnology*, 2000, vol. 2, 101-105
- Napoli, F., Olivieri G., Marzocchella A., Salatino P., "An Assessment of the Kinetics of Butanol Production by *Clostridium acetobutylicum*". *Int. J. Chemical Reactor Engineering*, 2009, vol.7, A45
- Nishio, N., H. Biebl, and M. Meiners, "Effect of pH on the production of acetone and butanol by *Clostridium acetobutylicum* in a minimum medium", *J. Ferment. Technol.* 1983, vol. 61, 101-104.
- Qureshi, N. and H.P. Blaschek, "Butanol production from agricultural biomass" *Food Sci. Biotechnol.*, 2005, 525-551
- Qureshi, N., A.H.J. Paterson and I.S. Maddox, "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.*, 1988, vol. 29, 323-328
- Tang, I.C., M.R. Okos and S.T. Yang, "Effects of pH and acetic acid on homoacetic fermentation of lactate by *Clostridium formicoaceticum*." *Biotechnology and Bioengineering.*, 1989, vol.34, 1063-1074
- Tashiro, Y., K. Takeda, G. Kobayashi and K. Sonomoto, "High production of acetone-butanol-ethanol with high cell density culture by cell-recycling and bleeding", *J. Biotechnol.*, 2005, vol. 120, 197-206
- Villadsen, J., "Innovative technology to meet the demands of the white biotechnology revolution of chemical production", *Chem Engng Sci.*, 2007, vol. 62, 6957-6968
- Welsh, F.W. and I. Veliky, "Production of acetone-butanol from acid whey". *Biotechnol. Lett.*, 1984, vol. 6, 61-64

- Yang, X. and G.T. Tsao “Mathematical modeling of inhibition kinetics in acetone-butanol fermentation by *Clostridium acetobutylicum*” Biotechnol. Prog., 1994, vol. 10, 532-538
- Yerushalmi, L., B. Volesky and J. Votruba, “Modelling of culture kinetics and physiology for *Clostridium acetobutylicum*”, The Canadian Journal of Chemical Engineering, 1986, vol. 64, 607-616
- Zeng, A.P. and W.D. Deckwer, “A model for multiproduct inhibited growth of *Enterobacter aerogenes* in 2,3-butanediol fermentation”, Appl. Microbiol. Biotechnol, 1991, 35, 1-3.
- Zeng, A.P., A. Ross, H. Bebl, C. Tag, B. Gunzel and W.D. Deckwer “Multiple product inhibition and growth modeling of *Clostridium butyricum* and *Klebsiella pneumoniae* in glycerol fermentation”, Biotechnology and Bioengineering, 1994, vol. 44, 902-911.

Table 1 A representative set of data measured during tests carried out in the CSTR at pH=5.

D= μ (h ⁻¹)	AA (mg/L)	BA (mg/L)	L (g/L)	δ (eq. 2) %
0.22	415	812	1.1	4
0.49	284	559	2.5	3
0.76	254	529	22.4	4
0.44	317	592	2.6	2
0.71	135	247	12.7	5
0.71	138	219	12.9	3
0.74	169	434	23.8	1
0.71	158	289	41.0	7

Table 2 Data measured during tests carried out in the CSTR at pH=5. Medium supplemented with acetic and butyric acids.

Supplemented product	D= μ (h ⁻¹)	AA (mg/L)	BA (mg/L)	L (g/L)	δ (eq. 2) %
Acetic acid	0.32	815	778	11.5	3
“	0.24	1050	250	7.1	4
Butyric acid	0.38	269	1411	12.7	6
“	0.15	264	2250	5.2	2
None	0.05	1088	2262	6.2	3
“	0.12	946	1994	8.8	4
“	0.15	758	1880	44.0	10
“	0.33	438	813	12.0	2
“	0.50	291	759	40.8	5
“	0.56	269	668	22.2	2
“	0.63	180	391	12.2	1

Table 3. Data measured during tests carried out in the CSTR at pH=5. Medium supplemented with solvents.

Supplemented product	D= μ (h ⁻¹)	AA (mg/L)	BA (mg/L)	B (mg/L)	Et (mg/L)	Ac (mg/L)	L (g/L)	δ (eq.2) %
Butanol	0.51	276	549	3072	-	-	11.7	3
“	0.52	199	461	6326	-	-	12.2	5
“	0.24	563	1202	5838	-	-	9.0	5
“	0.26	442	1023	8958	-	-	10.9	1
“	0.27	357	892	12082	-	-	10.7	2
“	0.19	292	679	14641	-	-	10.3	2
“	0.47	317	502	5363	-	-	6.7	5
“	0.32	239	429	11878	-	-	11.8	3
Acetone	0.36	460	938	-	-	5076	11.4	2
“	0.20	659	1390	-	-	14892	9.5	3
“	0.20	572	1222	-	-	25211	9.4	2
Ethanol	0.49	300	558	-	486	-	13.1	3
“	0.45	265	525	-	970	-	11.3	2
“	0.47	274	564	-	1854	-	14.0	1
“	0.47	299	625	-	5797	-	14.8	1
“	0.25	488	1019	-	11213	-	12.6	1
“	0.23	364	729	-	20702	-	12.2	1

Table 4 Data measured during tests carried out in the CSTR. pH effect

pH	D= μ (h ⁻¹)	AA (mg/L)	BA (mg/L)	L (g/L)	δ (eq. 2) %
4.0	0.24	383	800	12.5	2
“	0.32	118	260	14.0	4
4.2	0.12	900	1943	8.7	2
“	0.18	839	2061	9.0	3
“	0.35	542	1222	10.8	3
“	0.52	213	498	13.1	4
4.5	0.09	708	2276	8.1	4
“	0.27	635	1401	10.8	3
“	0.51	348	768	12.8	4
6.0	0.06	920	2234	4.9	4
“	0.30	493	871	10.7	3
“	0.55	205	450	13.1	3
“	0.60	69	113	6.4	2
7.0	0.10	80	286	7.7	2

Table 5 Model parameter of eq.s (8) and (16)

$\bar{\mu}_{\max} = 1.06 \text{ h}^{-1}$	$K_S = 1.34 \text{ g/L}$
$AA = 1.56 \text{ g/L}$	$n^{AA} = 0.98$
$BA = 3.00 \text{ g/L}$	$n^{BA} = 0.96$
$B = 16.50 \text{ g/L}$	$n^B = 0.47$
$Ac = 69.00 \text{ g/L}$	$n^{Ac} = 1$
$Et = 38.50 \text{ g/L}$	$n^{Et} = 1$
$K_H = 3.33 \cdot 10^{-4} \text{ M}$	$K_{OH} = 5.04 \cdot 10^{-7} \text{ M}$
$pH_{OPT} = 4.9$	$\Delta pH = 1.5$

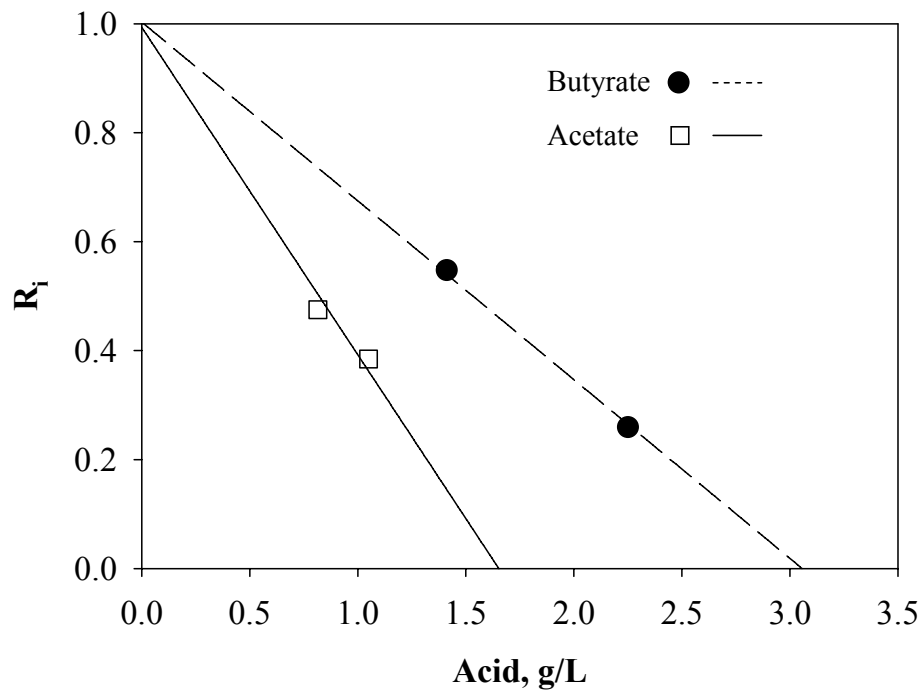


Figure 1 Inhibitory effect of supplemented acids on continuous culture of *C. acetobutylicum* DSMZ 792. pH=5.0. Lines represent plots of eq. (11).

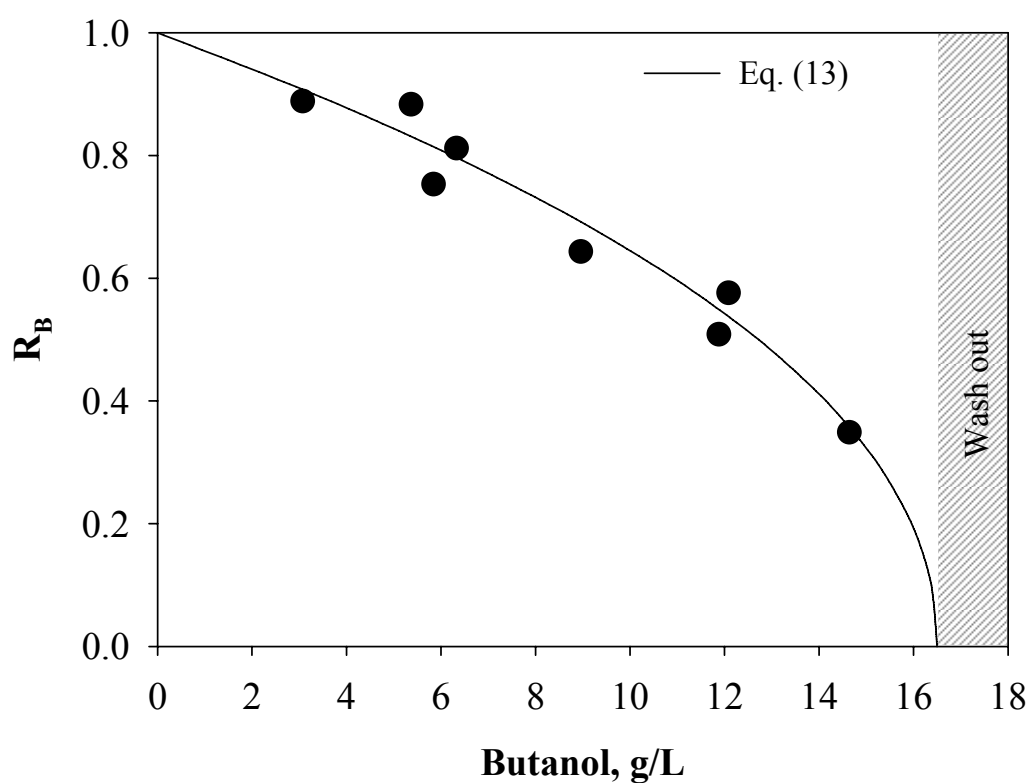


Figure 2 Inhibitory effect of butanol on continuous culture of *C. acetobutylicum*. pH=5

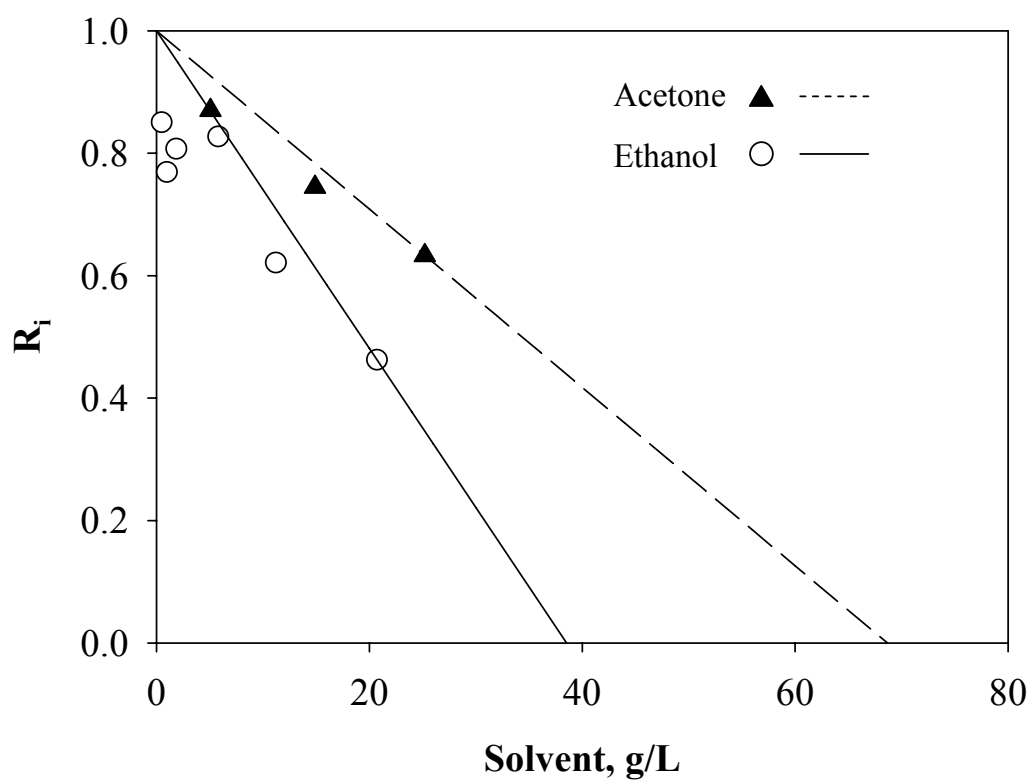


Figure 3 Inhibitory effects of ethanol and acetone on continuous culture of *C. acetobutylicum* DSMZ 792. pH=5. Lines represent plots of eq. (13).

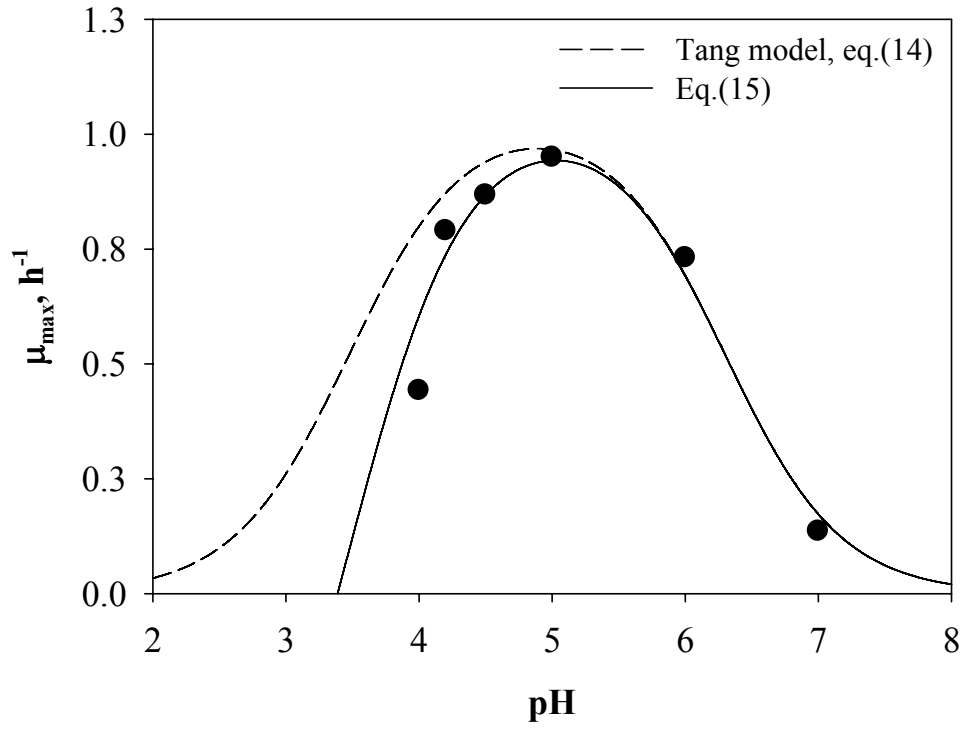


Figure 4 Maximum specific growth rate of *C. acetobutylicum* DSMZ 792 as a function of pH.

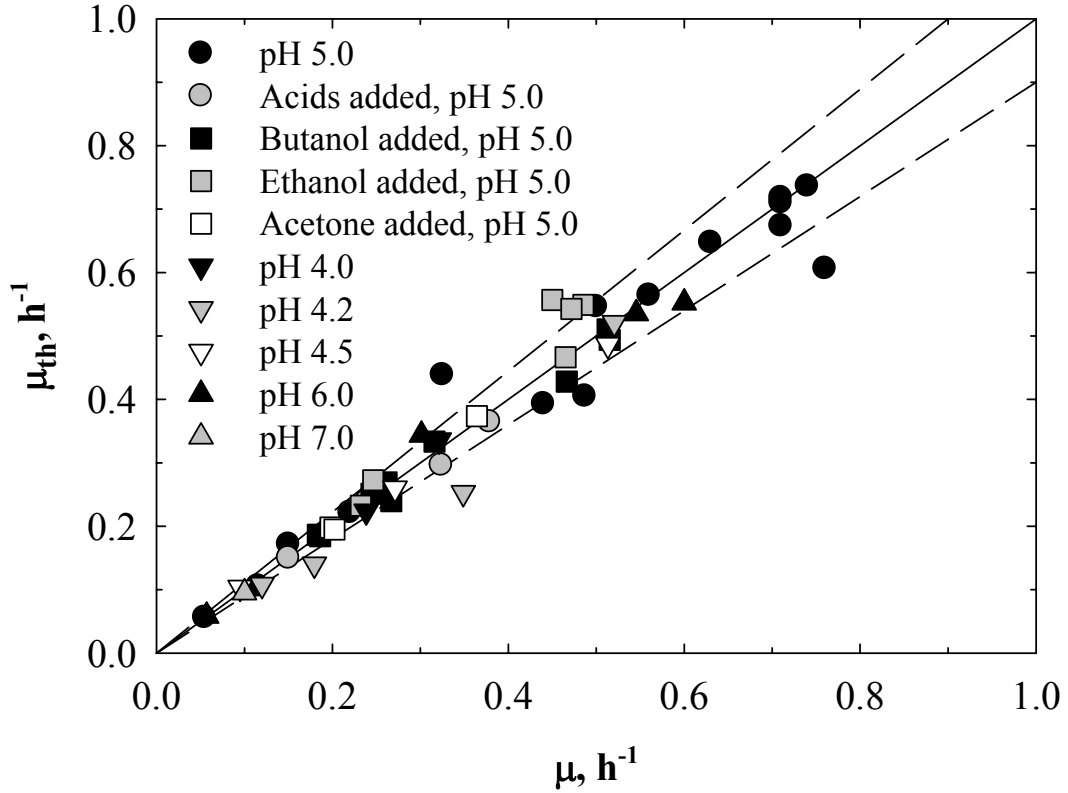


Figure 5 Measured vs predicted specific growth rate of *C. acetobutylicum* DSMZ 792
Dashed lines: $\pm 10\%$ error.

4.2 Butanol Production by *Clostridium acetobutylicum* – II. Mass and Energy Yields under Acidogenesis Phase

Fabio Napoli, Giuseppe Olivieri, Maria Elena Russo, Antonio Marzocchella, Piero Salatino

Chemical Engineering Department
School of Biotechnological Sciences – School of Engineering
Università degli Studi di Napoli *Federico II*
P.le V. Tecchio n. 1, Napoli – Italy

The growth of *Clostridium acetobutylicum* DSM 792 during acidogenesis phase under controlled conditions was investigated. A simplified stoichiometric growth model has been proposed. The model should complement the kinetic study reported in Part I (Napoli et al., 2009a). Tests were carried out in a continuous stirred tank reactor equipped with a pH-controller. Synthetic medium was used supplemented with lactose as carbon source. Acids and solvents - typically self-produced - were eventually supplemented to investigate their inhibitory effects on the products yield. The mass fractional yield of biomass and products were expressed as a function of the specific growth rate taking into account the Pirt model regarding the cell maintenance. Quantitative features of the *C. acetobutylicum* growth model were in satisfactory agreement with the experimental results. The model proposes as a tool to estimate the mass fractional yield even for fermentations carried out under conditions typical of the solventogenic phase.

Keywords: ABE, *Clostridium acetobutylicum*, continuous culture, ATP, yield, growth model.

INTRODUCTION

The production of biofuels by the biotechnological path is gaining a wide attention in the last decade for several issues, from eco-sustainability to the socio-economic aspects. In particular, biobutanol appears a potential substitute as fuel and solvent (Cascone, 2008). However, the industrial scale-up of the Acetone-Butanol-Ethanol (ABE) fermentation process still calls for investigation.

As extensively reported by Napoli et al. (2009a – named hereafter as Part I), there is still a lack of information in the literature supporting scale up and demonstration at the industrial scale. In particular, information on yield of substrate conversion in cells and butanol is extremely poor (Aiba and Shoda, 1969; Jones and Woods, 1986). In a recent review Villadsen (2007) pointed at the ABE process as an example of how far a more fundamental based approach could make process development more effective and successful.

Among the issues relevant for the competitiveness of butanol production processes, the biomass and product yields assume a key role. In fact, a streamlined production process should expect the conversion of the feedstock to the biofuel at the maximum possible values. Therefore, the assessment of the biomass and product yields is a prerequisite for the optimized design of biofuel production processes.

A sound tool to assess the biomass and product yields in a fermentation process may be based on the mass balances coupled with the ATP flow. In agreement with Bauchop and Elsdon (1960), a fermentation process may be characterized in terms of ATP yields (Y_{ATP}), expressed as dry mass of cells produced per mole of ATP generated. They proposed that the Y_{ATP} is 10.5 g_{DM}/mole_{ATP}, whatever the microorganism. On the other hand, Stouthamer (1973) estimated the Y_{ATP} on theoretical basis taking into account the ATP necessary for the formation of microbial cells and the energy adsorbed by transport phenomena through the cells membrane. They concluded that the upper bound of the ATP yield (Y_{ATP}^{MAX}) ranges between

28.6 and 32.1 g_{DM}/mole_{ATP} for microorganism growing on glucose and mineral salts.

For a given microorganism, Y_{ATP}^{MAX} may change with the specific growth rate as well as with the media composition (Forrest and Walker, 1971; Stouthamer and Bettenhausen, 1973). In particular, Pirt (1965) proposed the first relationship between Y_{ATP} and the specific growth rate (μ):

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

Where m is the maintenance coefficient, expressed as mol of ATP consumed for mass unit of dry cells formed. The m was defined “as the energy necessary for the turnover of substance and other cell components and for the preservation of the right ionic composition of the cells” (Stouthamer, 1973). A reliable relationship between m and μ was still proposed by Pirt (1982):

$$m = m_0 + m_1 \left(1 - \frac{\mu}{\mu_{max}} \right) \quad (2)$$

Where μ_{max} is the maximum specific growth rate, m_0 and m_1 the maintenance coefficient at μ equal to μ_{max} and 0, respectively.

With reference to the ABE production process, the assessment of the biomass and product yields tangles. The fermentation of *clostridia* strains – microorganisms widely adopted for ABE production – proceeds along a two phase process: *acidogenesis phase*) characterized by cells and acids production; *solventogenesis phase*) characterized by solvents production. Moreover, under solventogenesis phase a fraction of cells may produce acids, then cells (Jones and Woods, 1986; Meyer and Papoutsakis, 1989; Napoli *et al.*, 2009b). The co-presence of solventogenic and acidogenic cells under solventogenesis phase makes the assessment of both the biomass and the solvent yields a hard issue. Therefore, a reliable strategy may be the preliminary assessment of the biomass yield under a wide interval of operating conditions, eventually extended to those typical of the solventogenic phase. As a second step, the assessment of the solvents yield may take the advantage of the availability of the biomass yield model.

The present contribute reports the assessment of Y_{ATP} for acidogenic cells of *Clostridium acetobutylicum* DSMZ 792 adopting lactose as carbon source, with the aim of emulating cheese whey. The contribution is coupled with the investigation reported in Part I (Napoli *et al.*, 2009a). Data of biomass and acid concentration measured during batch and continuous cultures were worked out to assess the Y_{ATP} . The study addressed to the effects of acids (acetic and butyric), solvents (acetone, ethanol and butanol) and the pH on Y_{ATP} of *C. acetobutylicum* under acidogenic state. Accordingly, a model is proposed to describe acidogenic cells behaviour even under high ABE concentrations typical of solventogenic conditions, a mandatory step in the characterization of *C. acetobutylicum*.

EXPERIMENTAL

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Reactivation procedure, stock medium and culture medium are reported extensively in Part I.

Apparatus, diagnostics, procedures and operating conditions are reported in Part I.

THEORETICAL FRAMEWORK

Figure 1 shows a possible metabolic pathway of lactose by *C. acetobutylicum* under acidogenesis phase, in agreement with Bailey and Ollis (1986) and Jones and Woods (1986). The lactose diffuses in the cells and is cleaved by β -Galactosidase in two monomers: glucose and galactose. The glucose is oxidized to pyruvate through the well known Embden–Meyerhof–Parnas (EMP) pathway. The galactose is converted to glucose 6-phosphate by

phosphoglucumutase coupled with the reduction of ATP (1 mole of ATP to ADP for mole of galactose). The glucose 6-phosphate by *phosphoglucumutase* is then oxidized to pyruvate in agreement with the EMP pathway.

The comparison of the oxidization steps of glucose and galactose to pyruvate highlights that the two pathways are almost equivalent in terms of mass and energy balances. The bioconversion of lactose may be considered corresponding to the conversion of two molecules (mole) of glucose. The contribution of the glycolytic step to both the mass and energetic balances is: for each mole of lactose consumed along the EMP pathway, 4 moles of pyruvate and 4 moles of ATP are produced and 4 moles of NAD^+ are reduced to NADH_2 . All together, it is possible to assume that 1 mole of lactose is equivalent to 2 moles of glucose.

The pathway of the glucose conversion by *C. acetobutylicum* has been widely studied (Jones and Woods 1986; Doelle, 1975; Gottschalk, 1979; Haggstrom, 1985; Hartmanis and Gatenbeck, 1984; Rogers, 1984). The pyruvate is converted to Acetyl-CoA by pyruvate-ferrodoxin oxidoreductase producing CO_2 and H_2 . Provided acidogenic conditions, Acetyl-CoA may be converted to acetic and/or butyric acids coupled with production of ATP (Papoutsakis, 1973; Jones and Woods, 1986; Bailey and Ollis, 1986).

The ATP produced during both glicolysis and acidogenesis is booked for growth and maintenance of cells. The biomass yield, Y_{ATP} , may be assessed experimentally taking into account the biomass and metabolites produced, provided the stoichiometric relationship between metabolites and mole of ATP produced.

Equations (T.1.1) through (T.1.3) of table 1 summarize the formation reactions of biomass and metabolites during the acidogenesis phase of the lactose fermentation by *C. acetobutylicum*. For sake of simplicity, the ADP has been omitted where present.

With reference to the eq. (T.1.1), the biomass has been assumed as $\text{C}_4\text{H}_7\text{O}_2\text{N}$ and is characterized by: molecular weight $\text{MW}_x=101$ g/mol; carbon fraction $\alpha_c=0.475$; reductance degree $\gamma=4$. The elemental composition of the biomass has been set in agreement with a previous work carried out by Zeng *et al.* (1996) on *C. butylicum*. It should be highlighted that the elemental composition of the microorganism reported in the literature ranges over a quite wide interval. In particular, Papoutsakis (1983) and Andrews (1989) have suggested that γ and α_c , two important biological regularities, are typically equal to 4.262 ± 0.172 and 0.462 ± 0.023 , respectively. As a consequence of the values of γ and α adopted in eq. (T.1.1), the formation of the biomass does not require the oxidation of NADH_2 .

The molecular hydrogen is produced by oxidation of reduced ferrodoxin, under the assumption that all NADH_2 in excess is cleaved to H_2 .

The ATP production coupled with acids formation take into account the contribution of both the glycolytic pathway - lactose to pyruvate - and the acids formation pathway from *acetyl-CoA*. The energetic contribution is completely committed to the cell biosynthesis. Therefore, the ratio between the biomass and the acids produced reports a measure of the Y_{ATP} . Moreover, provided the ratio between butyric and acetic acids the exact value of Y_{ATP} may be assessed.

The value of the specific growth rate μ and of the $Y_{X/A}$ ratio may be assessed working out data measured during either continuous or batch cultures of *C. acetobutylicum*.

Batch cultures are characterized by exponential growth of cells and eq.s (3) and (4) hold:

$$\mu = \frac{\ln \frac{X}{X_0}}{(t - t_0)} \quad (3)$$

$$Y_{X/A} = \frac{X - X_0}{A - A_0} \quad (4)$$

Where t_0 and t are the beginning and a generic instant of the exponential growth phase, X_0 , X , A_0 and A the cell and the acids concentrations at t_0 and t , respectively.

Continuous cultures. With reference at acidogenic cultures carried out in chemostat, under the assumption of steady state conditions and sterile feed, the cell balance on CSTR is reduced to:

$$D = \mu \quad (5)$$

Where D is the dilution rate, the ratio between the volumetric flow rate of the feed and the reaction volume. The $Y_{X/A}$ yield may be assessed as:

$$Y_{X/A} = \frac{r_X}{r_A} = \frac{D \cdot X}{D \cdot (A - A_0)} = \frac{X}{(A - A_0)} \quad (6)$$

Where r_X and r_A are the mass production rate of biomass and acids, respectively, X the cell concentration in the reactor, A and A_0 the acids concentration in the reactor and in feed, respectively.

The theoretical molar uptake rate of the lactose (\dot{r}_L) under acidogenesis phase may be assessed in agreements with eq.s (T.1.1) through (T.1.3) and reads:

$$\dot{r}_L = \frac{\dot{r}_{AA}}{4} + \frac{\dot{r}_{BA}}{2} + \frac{\dot{r}_X}{3} \quad (7)$$

Where \dot{r}_i is the molar production rate of the metabolite “i”. Working out the eq. (3) under steady state conditions yields the theoretical consumed lactose (ΔL_{th}):

$$\Delta L_{th} = \left(\frac{\Delta AA}{4 \cdot MW_{AA}} + \frac{\Delta BA}{2 \cdot MW_{BA}} + \frac{\Delta X}{3 \cdot MW_X} \right) MW_L \quad (8)$$

where ΔAA , ΔBA and ΔX are the concentration increment – reactor concentration with respect to the feeding concentration - of acetic acid, butyric acid and cells, respectively, and MW_i the molecular weight of the specie “i”. The difference between theoretical value and the measured value of lactose consumed under steady state conditions has been expressed in term of the error δ :

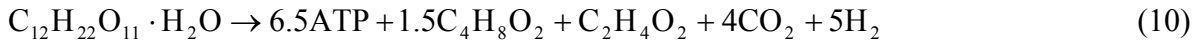
$$\delta = \frac{|\Delta L_{th} - \Delta L|}{\Delta L_{th}} \quad (9)$$

RESULTS and DISCUSSION

Tables 2 and 3 report data measured during tests carried out with the apparatus described in Part I. The tests were carried under a wide interval of operating conditions: lactose concentration in the feeding stream ranged between 4 and 50 g/L, D between 0.05 and 0.77 h⁻¹, and pH between 4.0 and 7.0. Some tests were carried out supplementing the lactose bearing stream with solvents in order to investigate the typical conditions of solventogenesis phase.

The figure 2 reports the molar ratio between butyric and acetic acids as a function of the dilution rate. The molar ratio is 1.5 (2.2 on mass basis) whatever the operating conditions adopted with a standard deviation of 0.17. The average value of the ratio is in agreement with data measured during tests carried out under batch conditions (Napoli et al., 2009b). Therefore, it is possible to affirm that the production rate of butyric acid (eq. T.1.3) is threefold acetic acid rate (eq. T.1.2). On the other hand, the analysis of eq.s (T.1.2) and (T.1.3) highlights that the acetic acids formation appears energetically favoured: the ATP yield with respect to the lactose is higher for the acetic acid formation. All together, reported results suggest that the acetic acid has an inhibitory effect stronger than the butyric acid. This observation is in agreement with the inhibitory concentration assessed for both acids: the inhibitory concentration of butyric acid is higher than that of acetic acid (Part I).

Assuming fixed the molar ratio between butyric and acetic acids at 1.5, the linear combination of eq.s (T.1.2) and (T.1.3) yields:



In particular, it results:

- the molar fractional yield ATP/lactose is 6.5;
- The molar ratio H_2/CO_2 is equal to 1.25;
- The mass of acids produced for mole of ATP produced ($Y_{A/ATP}$) is 29.5 g_{acids}/mole_{ATP}.

The value of Y_{ATP} may be assessed working out the value of $Y_{X/A}$ (fractional mass yield ratio between dry cells and total acids produced) and taking into account the value of $Y_{A/ATP}$:

$$Y_{ATP} = Y_{A/ATP} \cdot Y_{X/A} = 29.5 \cdot Y_{X/A} \quad (11)$$

Table 2 reports the values of $Y_{X/A}$, Y_{ATP} and ΔL_{th} assessed for continuous cultures carried out at pH=5.0 and at dilution rate ranging between 0.05 and the wash-out onset. Data of Y_{ATP} are reported in fig. 3 as a function of the specific growth rate assessed in agreement with eq. (5). The relative error between the theoretical and experimental values of ΔL was typically within 9%, highlighting the soundness of the assumptions.

The figure 3 shows the plot – dashed line - of eq. (1) assuming the maintenance coefficient constant with the specific growth rate (Pirt, 1965). The Y_{ATP}^{MAX} and “m” were set at 23.4 g_{DM}/mole_{ATP} and 0.0087 mole_{ATP}/h g_{DM}, respectively, that is at values averaged between results reported by Bahl *et al.* (1982), 23.8 g_{DM}/mole_{ATP} and 0.0035 mole_{ATP}/h g_{DM}, and Meyer and Papoutsakis (1989), 23 g_{DM}/mole_{ATP} and 0.014 mole_{ATP}/h g_{DM}. The comparison between the experimental data and the Pirt model (1965) is quite poor, when data at high μ are contemplated. The regression of data in table 2 accordingly to eq. (1) yields a value of Y_{ATP}^{MAX} of about 40 g_{DM}/mole_{ATP}, larger than the maximum theoretical value (Stouthamer, 1973).

Data of Y_{ATP} were worked out in agreement with eq.s (1) and (2). Assuming m_0 negligible, the combination of eq.s (1) and (2) yields:

$$Y_{ATP} = Y_{ATP}^{MAX} \frac{\mu}{\mu + Y_{ATP}^{MAX} \times m_1 \left(1 - \frac{\mu}{\mu_{max}} \right)} \quad (12)$$

The regression of data reported in table 2 in agreement with eq. (12) and assuming $\mu_{max}=1.06 \text{ h}^{-1}$ (Part I) yields:

$$Y_{ATP}^{MAX}=29.1 \pm 1 \text{ g}_{DM}/\text{mole}_{ATP} \quad m_1=0.012 \pm 0.0008 \text{ mole}_{ATP}/\text{h g}_{DM} \quad (13)$$

The plot of eq. (12) is also reported in fig. 3 and the agreement with experimental data is satisfactory, supporting the reliability of the model proposed.

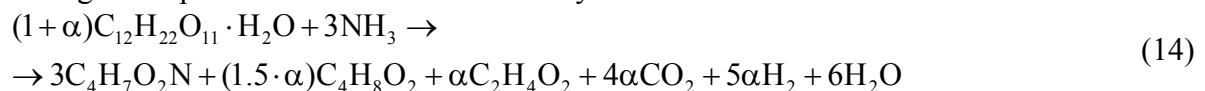
Effects of pH and solvents on Y_{ATP}

Table 3 reports data of tests aimed at investigating the effects of the pH and of the solvents on the Y_{ATP} . In particular, the pH was set at values ranging between 4.0 and 7.0 and acetone, butanol and ethanol - solvents typically produced by *C. acetobutylicum* under solventogenesis phase (Part I) - were eventually supplemented to the lactose bearing stream.

The value of Y_{ATP} assessed accordingly with eq. (12) are reported in table 2 and plotted in fig. 4 as a function of the specific growth rate. The agreement between experimental data and the plot of eq. (12) adopting the parameters (13) is satisfactory.

Products fractional yield

The eq.s (T.1.1) and (10) may be combined assuming that the ATP produced during acidogenesis phase is committed to cell biosynthesis:



Where

$$\alpha = \frac{3 \frac{MW_X}{Y_{ATP}}}{6.5} = 46.62 / Y_{ATP} \quad (15)$$

The eq. (14) becomes a tool to estimate the acidogenic cells and the acids fractional yields as a function of the specific growth rate.

The data reported in tables 1 and 2 have been worked out and reported in fig. 5 as mass fractional yield of biomass ($Y_{X/L}$), butyric acid ($Y_{BA/L}$) and acetic acid ($Y_{AA/L}$) as a function of the specific growth rate. The theoretical values of the yields estimated accordingly with eq.s (12) and (14) adopting the parameters (13) are also reported in fig. 5. The agreement between the experimental data and the estimated value of the yield is satisfactory with an average relative error equals to 6, 7 and 10% for $Y_{X/L}$, $Y_{BA/L}$ and $Y_{AA/L}$, respectively.

Batch tests

The table 3 reports data measured during batch tests carried out at initial lactose concentrations ranging between 10 and 110 g/L (after Napoli et al., 2009b). The specific growth rate, assessed in agreement with eq. (3), did not change significantly because the initial lactose concentration is greater than the saturation constant (1g/L) of the growth Monod model. On the other hand, the Y_{ATP} assessed in agreement with eq.s (4) and (11) changed between 10.6 and 15.6. The comparison with the Pirt's model eq.s (12)-(13) is not satisfactory. The disagreement between the experimental and assessed data together with the high value of the error δ suggests that the model can not apply to batch cultures. The issue asks for more investigations.

Acids yield

The acids conversion under solventogenic conditions may proceed at a rate different for the acetic and the butyric component. In particular, the ratio between butyric and the acetic acids under steady state solventogenic conditions may be larger or smaller than 1.5 found under acidogenic conditions. The results available in literature on this issue are quite contradictory. Ruchir *et al.* (1999) pointed out that *in vitro* the rate of the acetic ac. uptake is threefold the rate of butyric ac. uptake. On the other hand, Mermelstein *et al.*, (1993) reported that the butyric ac. uptake is more rapid than that of acetic ac. Therefore, more information is necessary to address the assessment of the overall ABE production rate.

The “net” production of acids under operating conditions close to the solventogenic phase has been assessed by means of the Y_{ATP} model proposed. In particular, the experimental and the theoretical value of Y_{ATP} have been assessed for tests carried out supplementing acids to the lactose bearing stream.

The assessment of the $Y_{X/A}$ referred to runs carried out supplementing an acid to the feeding is made difficult by the inhibitory effect of the acid itself. On the other hand, the measurement of the concentration increment of the acid supplemented as a consequence of its production during the fermentation may be quite unreliable (the increment may be nearby the measurement error). The imprecision may be remedied taking into account the molar ratio between butyric and acetic acids.

The $Y_{X/A}$ referred to a run carried out supplementing butyric acids to the lactose bearing stream may be estimated in agreement with the eq. (16):

$$Y_{X/A} = \frac{X}{\left(AA + 1.5 \frac{AA}{MW_{AA}} MW_{BA} \right)} \quad (16)$$

For runs carried out supplementing acetic acid eq. (17) holds:

$$Y_{X/A} = \frac{X}{\left(BA + \frac{1}{1.5} \frac{BA}{MW_{BA}} MW_{AA} \right)} \quad (17)$$

Where AA and BA are, respectively, the mass concentration of acetic ac. and butyric ac. produced under steady state conditions.

The table 5 reports data referred to tests carried out supplementing the lactose bearing stream with acids. Figure 6 shows the Y_{ATP} vs. μ assessed for the tests in table 5 and estimated by means of eq.s (12)-(13). The agreement is satisfactory and confirms the hypothesis of the molar ratio between the butyric and the acetic ac.s produced is equal to 1.5, even when the acids ratio in the fermenter is different from 1.5.

CONCLUSIONS

The growth of *Clostridium acetobutylicum* DSM 792 under acidogenic conditions using lactose as substrate was successfully investigated by means of a CSTR operated at pH constant. The pH ranged between 4.0 and 7.0. The lactose bearing stream were eventually supplemented with acetic acid, butyric acid, ethanol, acetone and butanol - solvents typically produced by the investigated microorganism under solventogenic phase – to investigate their role on the microorganism products yield.

A simplified stoichiometric growth model has been proposed. The model proposes as a tool to assess the mass fractional yield of both the biomass and the acids as a function of the operating conditions, expressed in terms of the specific growth rate (Part I). In particular, the maintenance model of Pirt (1982) was successfully adopted as a relationship between the specific growth rate and the energetic yield necessary for the biosynthesis.

The agreement between the theoretical and experimental yields was successfully over a wide interval of operating conditions. In particular, the model was able to estimate the yields even under extreme operating conditions: i) huge presence of acids, properly supplemented; ii) huge presence of solvents typically produced during the solventogenic phases. The latter conditions assume a relevant role in the investigation of the ABE production processes. In fact, under solventogenesis phase a fraction of cell produces biomass and acids.

NOMENCLATURE

A	total acids concentration; g/L
AA	acetic acid concentration; g/L
BA	butyric acid concentration; g/L
L	lactose concentration; g/L
m, m_0, m_1	maintenance coefficient of the Pirt model; mole _{ATP} /h g _{DM}
r_i	mass production/uptake rate of species “i”; g/Lh
\dot{r}_i	molar production/uptake rate of species “i”; mole/Lh
$Y_{BA/L}$	fractional yield of lactose in butyric acid; g/g
$Y_{AA/L}$	fractional yield of lactose in acetic acid; g/g
$Y_{X/A}$	mass ratio between biomass and total acids; g _{DM} /g
$Y_{X/L}$	fractional yield of lactose in biomass; g _{DM} /g
$Y_{A/ATP}$	ratio between total acids and ATP moles; g/mole _{ATP}
Y_{ATP}	ATP yield; g _{DM} /mole _{ATP}
Y_{ATP}^{MAX}	maximum ATP yield; g _{DM} /mole _{ATP}
X	cell concentration; g _{DM} /L

Greek Letters

α	stoichiometric coefficient
----------	----------------------------

α_C	biomass carbon fraction; g/g _{DM}
δ	relative error between theoretical and experimental lactose uptake
γ	reductance degree
μ	specific growth rate; h ⁻¹
μ_{\max}	maximum specific growth rate; h ⁻¹

Subscript

0	initial or feeding value
th	theoretical

REFERENCES

- Aiba, S. and M. Shoda, "Reassessment of product inhibition in alcohol fermentation" J. Ferment. Technol., 1969, vol. 47, 790-794
- Andrews, G., "Estimating cell and product yield", Biotechnology and Bioengineering, 1989, vol. 33, 256-265.
- Bahl, H., W. Andersch, K. Braun, and G. Gottschalk, "Effect of pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* grown in continuous culture", Eur. J. Appl. Microbiol. Biotechnol., 1982, vol. 14, 17-20
- Bailey, J.E., D.F. Ollis, *Biochemical Engineering Fundamentals*. McGraw-Hill, New York. 1986
- Bauchop, T. and S. R. Elsdon, "The growth of microorganisms in relation to their energy supply", J. Gen. Microbiol., 1960, vol. 23: 457-469
- Doelle, H. W. 1975. Bacterial metabolism, 2nd ed. Academic Press, Inc., New York.
- Ezeji, T.C., N. Qureshi and H.P. Blaschek, "Bioproduction of butanol from biomass: from genes to bioreactors", Curr. Op. Biotechnol., 2007, vol. 18, 220-227
- Forrest, W. W. and D. J. Walker, "The generation and utilization of energy during growth", Advan. Microb. Physiol., 1971, vol. 5, 213-274.
- Gottschalk, G. 1979. Bacterial metabolism. Springer Verlag, New York.
- Haggstrom, L. "Acetone-butanol fermentation and its variants", Biotechnol. Adv. 1985, vol. 3, 13-28.
- Hartmanis, M. G. N., and S. Gatenbeck, "Intermediary metabolism in *Clostridium acetobutylicum*: levels of enzymes involved in the formation of acetate and butyrate" Appl. Environ. Microbiol., 1984, vol. 47, 1277-1283
- Huang, W.C., D.E. Ramey and S.T. Yang, "Continuous production of butanol by *Clostridium acetobutylicum* immobilized in a fibrous bed", Bioreactor Appl. Biochem Biotechnol, 2004, vol. 113, 887-898
- Jones, D.T. and D.R. Woods, "Acetone-butanol fermentation revisited" Microb. Rev., 1986, Dec, 484-524
- Long, S., D.T. Jones and D.R. Woods, "Initiation of solvent production, clostridial stage and endospore formation in *Clostridium acetobutylicum* P262." Appl. Microbiol. Biotechnol., 1984, vol. 20, 256-261.
- Mermelstein, L.D., Papoutsakis, E.T., Petersen, D.J., Bennett, G.N., "Metabolic engineering of *Clostridium acetobutylicum* ATCC 824 for increased solvent production by the enhancement of acetone formation enzyme activities using a synthetic acetone operon", Biotechnol. Bioeng., 1993, vol. 42, 1053-1060.
- Meyer, C.L. and E.T. Papoutsakis, "Continuous and biomass recycle fermentations of *Clostridium acetobutylicum*. Part 1: ATP supply and demand determines product selectivity", Bioprocess Engineering, 1989, vol. 4, 1-10
- Napoli, F., Olivieri G., Russo M.E., Marzocchella A. and Salatino P., "Butanol Production by

- Clostridium Acetobutylicum* – I. Acidogenesis Kinetics”, Bioengineering and Biotechnology, 2009a, submitted
- Napoli, F., Olivieri G., Marzocchella A. and Salatino P., “An Assessment of the Kinetics of Butanol Production by *Clostridium acetobutylicum*”, Int. J. Chemical Reactor Engineering, 2009b, vol. 7, A45
- Qureshi, N. and H.P. Blaschek, “Butanol production from agricultural biomass” Food Sci. Biotechnol., 2005, 525-551
- Papoutsakis, E. T., “Equations and calculations for fermentations of butyric acid bacteria” Biotechnology and Bioengineering, 1984, vol. 26, 174-187
- Pirt, S. J., “The maintenance energy of bacteria in growing cultures”, Proc. Roy. Soc. London, . 1965, vol. 163B, 224-231.
- Pirt, S. J., “Maintenance energy: a general model for energy-limited and energy-sufficient growth”, Arch Microbiol., 1982, vol. 302, 133:300
- Rogers, P., “Genetics and biochemistry of *Clostridium* relevant to development of fermentation processes”, Appl. Microbiol., 1984, vol. 31, 1-60.
- Ruchir P., L. Desai, K. Nielsen, E.T. Papoutsakis, “Stoichiometric modeling of *Clostridium acetobutylicum* fermentations with non-linear constraints”, Journal of Biotechnology, 1999, vol. 71, 191–205.
- Stouthamer, A. H., “A theoretical study on the amount of ATP required for synthesis of microbial cell material”, Antonie van Leeuwenhoek, 1973, vol. 39, 545-565.
- Stouthamer A. H. and C. Bettenhausen, “Utilization of energy for growth and maintenance in continuous and batch cultures of microorganisms a reevaluation of the method for the determination of ATP production by measuring molar growth yields”, Biochimica et Biophysica Acta, 1973, vol. 301, 53-70
- Tashiro, Y., K. Takeda, G. Kobayashi and K. Sonomoto, “High production of acetone-butanol-ethanol with high cell density culture by cell-recycling and bleeding”, J. Biotechnol., 2005, vol. 120, 197-206
- Villadsen, J., “Innovative technology to meet the demands of the white biotechnology revolution of chemical production”, Chem Engng Sci., 2007, vol. 62, 6957-6968
- Zeng A.-P. “Pathway and kinetic analysis of 1,3-propanediol production from glycerol fermentation by *Clostridium butyricum*” Bioprocess Engineering, 1996, vol. 14, 169-175

Table 1. Formation reactions of biomass and metabolites: acidogenesis phase

Biomass	$C_{12}H_{22}O_{11} \cdot H_2O + 3NH_3 + 3 \frac{MW_x}{Y_{ATP}} ATP \rightarrow 3C_4H_7O_2N + 6H_2O$	(T.1.1)
Acetate	$C_{12}H_{22}O_{11} \cdot H_2O + 4H_2O \rightarrow 8ATP + 4C_2H_4O_2 + 4CO_2 + 8H_2$	(T.1.2)
Butyrate	$C_{12}H_{22}O_{11} \cdot H_2O \rightarrow 6ATP + 2C_4H_8O_2 + 4CO_2 + 4H_2$	(T.1.3)

Table 2. Data measured and assessed during continuous cultures under steady state conditions. Tests carried out at pH=5.0.

D (h ⁻¹)	Biomass (g _{DM} /L)	Lactose (g/L)	Acetic ac. (mg/L)	Butyric ac. (mg/L)	Y _{X/A} g/g	Y _{ATP} g _{DM} /mole	ΔL (g/L)	ΔL _{th} (g/L)	δ (%)
0.05	0.96	6.2	1088	2262	0.29	8.5	8.1	7.4	9%
0.12	0.84	8.8	946	1994	0.26	7.6	6.2	6.5	5%
0.15	1.02	44.0	758	1880	0.39	11.5	6.0	6.2	3%
0.18	0.95	8.4	765	1621	0.40	11.7	5.5	5.6	2%
0.22	0.59	1.1	415	812	0.48	14.2	3.5	3.0	17%
0.33	0.60	12.0	438	813	0.48	14.2	2.9	3.0	3%
0.44	0.48	2.6	317	592	0.53	15.6	2.0	2.3	11%
0.49	0.55	2.5	284	559	0.66	19.4	2.1	2.2	7%
0.50	0.72	40.8	291	759	0.69	20.2	2.8	2.8	0%
0.56	0.72	22.2	269	668	0.77	22.7	2.7	2.6	4%
0.63	0.48	12.2	180	391	0.84	24.8	1.4	1.6	13%
0.71	0.36	12.7	135	247	0.94	27.8	0.94	1.14	17%
0.71	0.30	12.9	138	219	0.84	24.8	0.70	1.01	31%
0.71	0.52	23.8	169	434	0.86	25.4	1.16	1.76	34%
0.74	0.40	34.0	158	289	0.89	26.4	2.0	1.3	53%
0.76	0.70	22.4	254	529	0.90	26.5	2.5	2.3	9%
0.77	0.20	13.9	86	174	0.77	22.6	-0.1	0.7	114%

Table 3. Data measured and assessed in continuous cultures under steady state conditions.
Effects of the pH and of solvents.

Product Supplemented (g/L)	pH -	D (h ⁻¹)	Biomass (g _{DM} /L)	Lactose (g/L)	Acetic ac. (mg/L)	Butyric ac. (mg/L)	Y _{X/A} g/g	Y _{ATP} (g _{DM} /mole)	ΔL (g/L)	ΔL _{th} (g/L)	δ (%)
None	4.0	0.24	0.62	12.5	383	800	0.52	15.5	1.30	0.72	219%
“	4.0	0.32	0.20	14.0	118	260	0.53	15.7	0.9	1.0	10%
“	4.2	0.12	0.77	8.7	900	1943	0.27	7.9	6.2	6.2	0%
“	4.2	0.18	1.30	9.0	839	2061	0.39	11.4	6.4	7.0	9%
“	4.2	0.35	1.18	10.8	542	1222	0.67	19.7	4.6	4.7	2%
“	4.2	0.52	0.61	13.1	213	498	0.86	25.2	2.1	2.1	0%
“	4.5	0.09	0.80	8.1	708	2276	0.27	7.9	6.4	6.7	4%
“	4.5	0.27	1.07	10.8	635	1401	0.52	15.5	4.5	5.1	12%
“	4.5	0.51	0.75	12.8	348	768	0.67	19.7	2.6	3.0	13%
“	6.0	0.06	0.58	4.9	920	2234	0.18	5.4	8.0	6.6	21%
“	6.0	0.30	0.65	10.7	493	871	0.48	14.0	4.3	3.3	30%
“	6.0	0.55	0.51	13.1	205	450	0.79	23.2	1.9	1.8	6%
“	6.0	0.60	0.12	6.4	69	113	0.65	19.3	0.5	0.5	0%
“	7.0	0.10	0.05	7.7	80	289	0.13	3.8	0.4	0.8	48%
Butanol (3.0)	5.0	0.51	0.63	11.7	276	549	0.76	22.5	2.6	2.3	13%
Butanol (5.0)	5.0	0.47	0.55	6.7	317	502	0.68	19.9	2.2	2.2	0%
Butanol (6.0)	5.0	0.52	0.53	12.2	199	461	0.80	23.6	2.0	1.9	5%
Butanol (6.5)	5.0	0.24	0.69	9.0	563	1202	0.39	11.5	4.6	4.1	12%
Butanol (9.0)	5.0	0.26	0.66	10.9	442	1023	0.45	13.4	3.7	3.5	6%
Butanol(11.0)	5.0	0.32	0.34	11.8	239	429	0.51	15.0	1.3	1.6	19%
Butanol(12.0)	5.0	0.27	0.60	10.7	357	892	0.48	14.1	2.7	3.1	13%
Butanol(14.5)	5.0	0.19	0.45	10.3	292	679	0.47	13.8	2.5	2.4	4%
Ethanol (0.5)	5.0	0.49	0.65	13.1	300	558	0.76	22.5	2.1	2.4	13%
Ethanol (1.0)	5.0	0.45	0.47	11.3	265	525	0.60	17.6	2.2	2.0	10%
Ethanol (2.0)	5.0	0.47	0.53	14.0	274	564	0.63	18.7	1.5	2.2	32%
Ethanol (6.0)	5.0	0.47	0.58	14.8	299	625	0.63	18.6	2.4	2.4	0%
Ethanol(11.0)	5.0	0.25	0.68	12.6	488	1019	0.45	13.2	3.3	3.6	8%
Ethanol(20.0)	5.0	0.23	0.45	12.2	364	729	0.42	12.2	2.6	2.6	0%
Acetone (5.0)	5.0	0.36	0.65	11.4	460	938	0.46	13.7	3.3	3.4	3%
Acetone(15.0)	5.0	0.20	0.82	9.5	659	1390	0.40	11.8	4.5	4.8	6%
Acetone(25.0)	5.0	0.20	0.68	9.4	572	1222	0.38	11.3	4.1	4.2	2%

Table 3 Data measured and assessed in batch cultures (after Napoli et al., 2009b).

Initial Lactose (g/L)	μ (h ⁻¹)	$Y_{X/A}$ g/g	Y_{ATP} g _{DM} /mole	ΔL (g/L)	ΔL_{th} (g/L)	δ (%)
10.5	0.27	0.48	14.1	3.9	3.8	3%
16.6	0.28	0.36	10.6	3.1	1.9	63%
49.5	0.27	0.53	15.6	3.9	4.7	17%
79.4	0.23	0.46	13.6	2.1	3.7	43%
108.9	0.27	0.37	10.9	2.9	2.6	12%

Table 5 Data measured and assessed in continuous cultures under steady state conditions. Effects of the acid supplemented to the feeding.

Product Supplemented (mg/L)	pH	D (h ⁻¹)	Biomass (g _{DM} /L)	Lactose (g/L)	Acetic ac. (mg/L)	Butyric ac. (mg/L)	Y _{X/A} g/g	Y _{ATP} gDM/mole	ΔL (g/L)	ΔL _{th} (g/L)	δ (%)
Acetate(500)	5.0	0.32	0.58	11.5	815	778	0.54	15.82	2.4	2.8	15%
Acetate(1000)	5.0	0.24	0.56	7.1	1050	250	0.64	12.7	0.8	0.9	8%
Butyrate(1000)	5.0	0.38	0.16	12.7	269	1411	0.43	18.8	1.9	2.3	18%
Butyrate(2000)	5.0	0.15	0.23	5.2	264	2250	0.29	8.6	1.7	1.9	9%

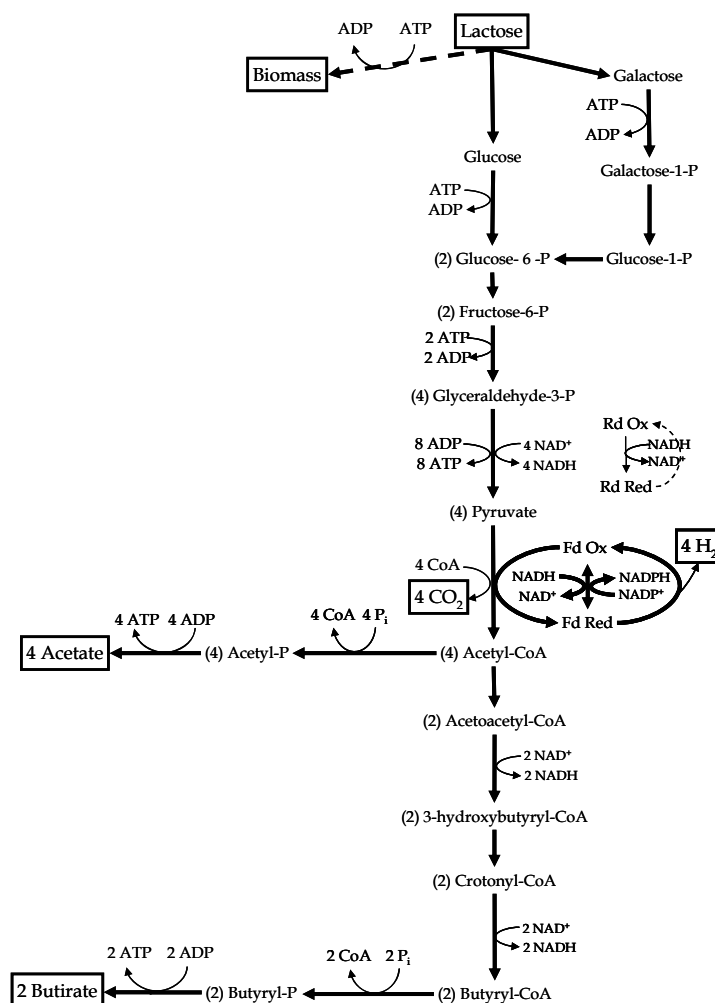


Figure 2. Metabolic acidogenesis pathways from lactose by *C. acetobutylicum*.

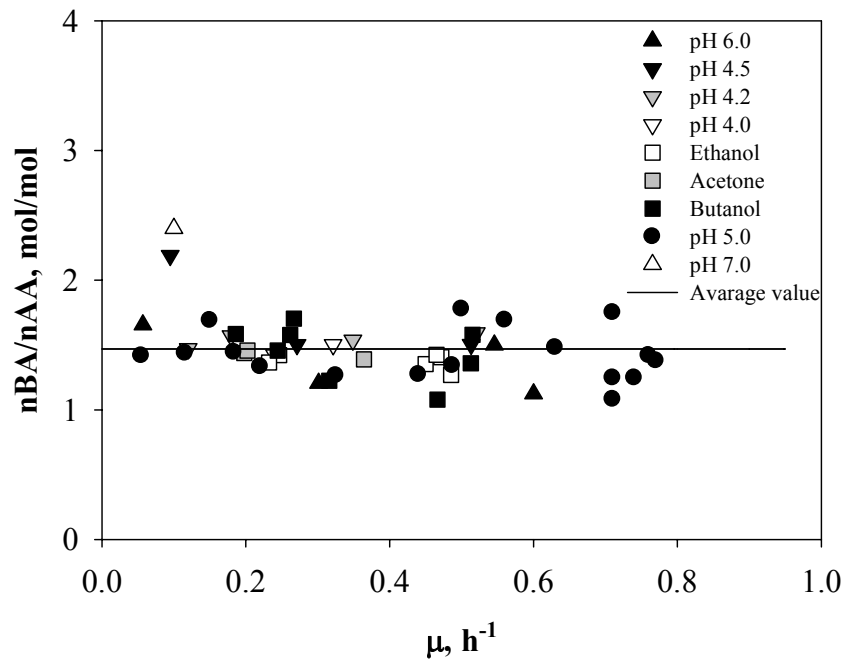


Figure 2. Molar ratio between butyric and acetic acids. Steady states under acidogenic conditions.

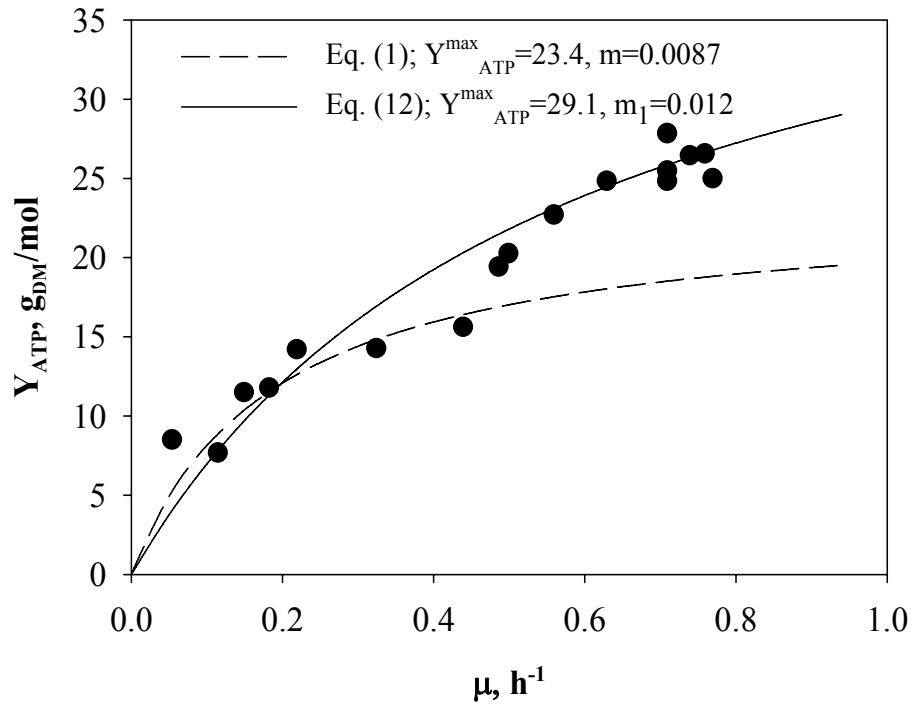


Figure 3. Y_{ATP} vs specific growth rate. Tests carried out at pH=5.0

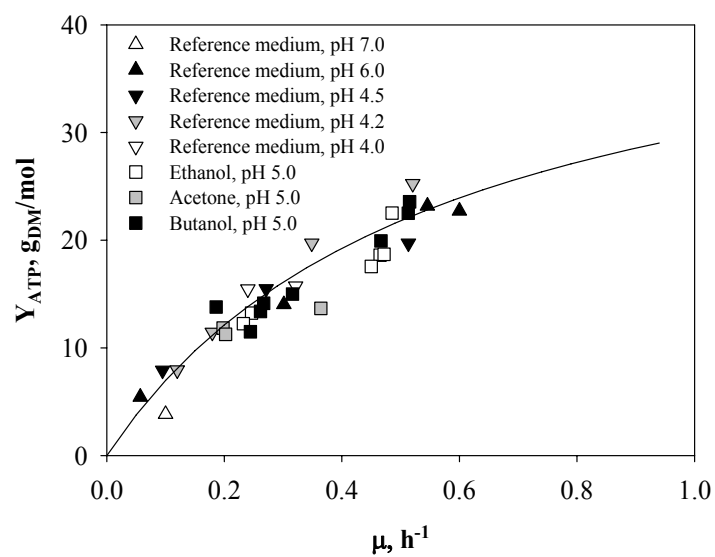


Figure 4. Y_{ATP} vs specific growth rate. Effects of pH and of solvents. The line is the plot of the theoretical values assessed in agreement with eq.s (12)-(13).

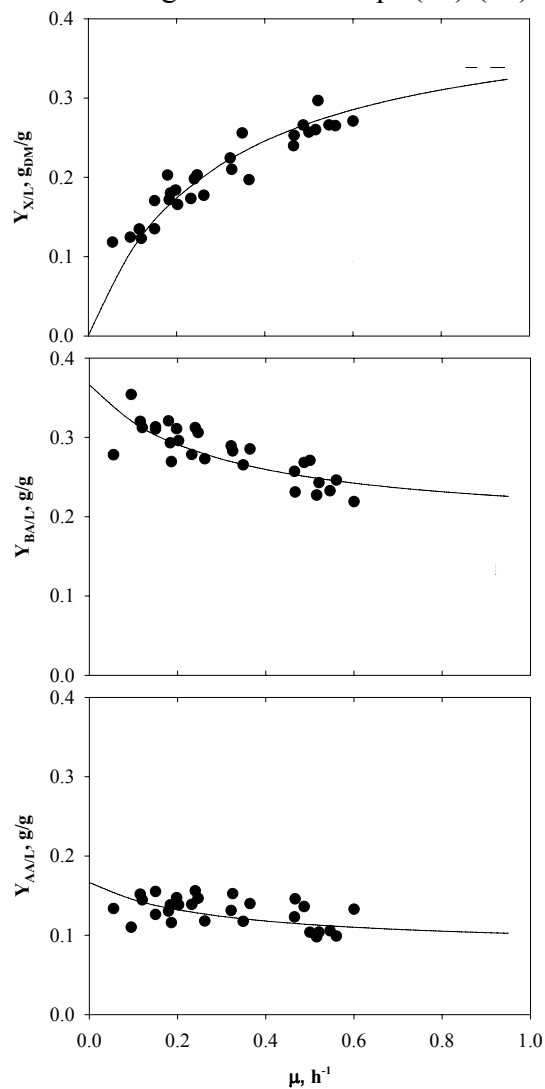


Figure 5. Fractional mass yield of cells and produced acids as a function of μ . Lines are plots of the theoretical values assessed in agreement with eq.s (12)-(14).

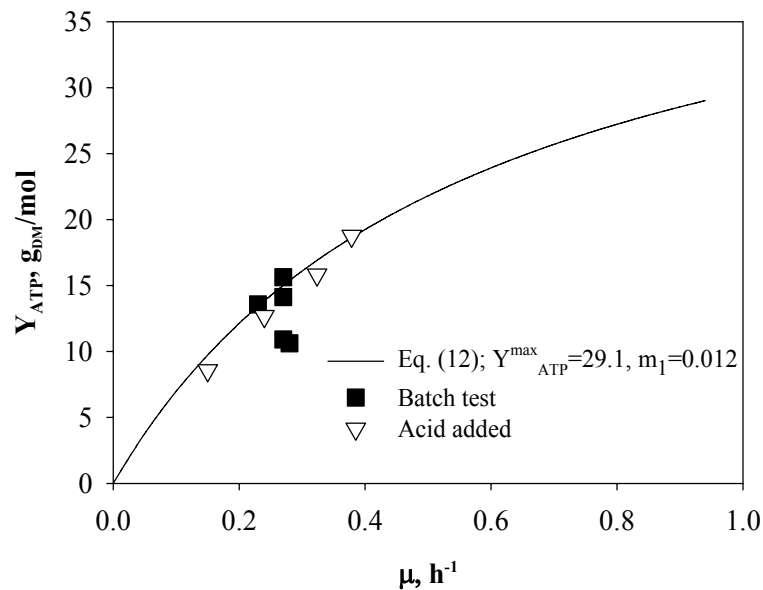


Figure 6. Y_{ATP} as a function of μ . Batch tests: Napoli et al. (2009b). Continuous tests: acids were supplemented to lactose bearing stream

4.3 Solventogenic Phase: Preliminary Tests and Data Analysis

The present section reports results on the characterization of the solventogenic phase. The investigation has been carried out in a continuous stirred tank reactor equipped with microfiltration unit, to confine the solventogenic cells in the reactor. The results have been analysed in the light of the theoretical framework reported in section § 3.1 and taking into account the kinetics and the growth characterization of the acidogenic phase reported in §4.2 and 4.3. The effect of pH on the ABE process has also been assessed in the range 4.0-4.5. The results have been worked out to assess the concentration of acidogenic cells (X_A), solventogenic cells (X_S) and spore (X_D). Furthermore, the process has been characterized in terms of both acetic and butyric acids uptake and direct production of butanol from lactose.

Material and methods

Microorganism and media

C. acetobutylicum DSMZ 792 and media composition, are described in §4.2.

Apparatus

The apparatus for the continuous bioconversion of lactose by free cells under solventogenic conditions is sketched in figure 3.1. Basically it was described in the paper § 3.1. It consisted of a thermostated CSTR equipped with a microfiltration unit and a pH controller.

The lactose bearing stream was fed at the volumetric rate (Q_0) set in accordance with the pre-set dilution rate ($D=Q_0/V_r$). The value of the volumetric rate Q_1 was set in order to prevent reactor wash-out ($D_1=Q_1/V_r$ smaller of the maximum specific growth rate). At the end of the run the microfiltration cartridge was washed to remove entrapped/immobilized cells and the recovered dry biomass weighted. The culture was periodically sampled to measure cell and metabolites concentrations. Data measured under steady state conditions were worked out to assess values of fractional yield coefficients, conversion degree and conversion/production rates.

The working volume of the bioreactor was $V_r=300$ mL volume, the cut-off of

the microfiltration cartridge was 0.22 μm . The pH was controlled adding a NaOH 1 M solution and measured on line by a pH probe insert in the reactor.

The reactor was operated continuously with respect to the liquid phase at a dilution rate $D=(d_1+d_2)=0.028\text{ h}^{-1}$, where $d_2=Q_1/V_r$.

Theoretical consideration

The model reported in the § 2.1 with reference to a CSTR equipped with a microfiltration unit may be adopted taking into account the constitutive equation for μ and Y_{ATP} reported in § 4.2 and § 4.3 respectively. Accordingly, the set of equation are simplified and reported in table 4.1. In particular, eq. (2.8) has been substituted by

Table 4.1 Simplified fermentation model for the system deccribed in Fig. 4.1

Acetic acid	$F_{\text{AA}} = f_1^{\text{AA}} - f_4^{\text{AA}}$	(T.4.1.1)
Butyric acid	$F_{\text{BA}} = f_2^{\text{BA}} - f_5^{\text{BA}}$	(T.4.1.2)
Butanol	$F_{\text{B}} = \frac{f_4^{\text{AA}}}{2} + f_5^{\text{BA}} + f_6^{\text{B}}$	(T.4.1.3)
Ethanol	$F_{\text{Et}} = f_7^{\text{Et}}$	(T.4.1.4)
Acetone	$F_{\text{Ac}} = f_4^{\text{AA}} + f_5^{\text{BA}}$	(T.4.1.5)
Butyric ac. - acetone ratio	$\frac{f_2^{\text{BA}}}{f_1^{\text{AA}}} = 1.5$	(T.4.1.6)
Biomass	$W_X = w_X^A + w_X^S + w_X^D$	(T.4.1.7)
Acidogenic biomass	$w_X^A = \mu X_A - \mu_S X_A$	(T.4.1.8)
Growth rate	$\mu = \mu_{(AA,BA,B,Ac,Et,L,pH)}$	(T.4.1.9)
Concentration	$X_A = \frac{Y_{\text{ATP}} _{(\mu)} \cdot (2f_1^{\text{AA}} + 3f_2^{\text{BA}})}{\mu}$	(T.4.1.10)
Solventogenic biomass	$w_X^S = \mu_S X_A - \mu_D X_S$	(T.4.1.11)
Specific rate of sporulation	$\mu_D = 0.0045$	(T.4.1.12)

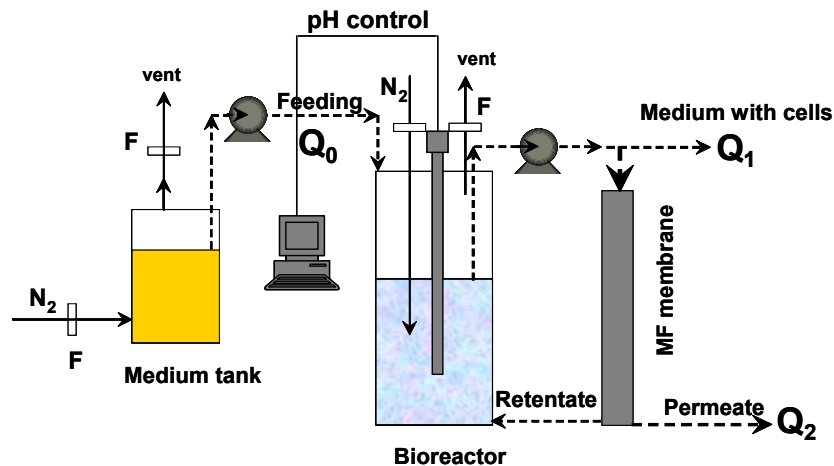


Figura 4.1 Apparatus for solventogenic tests.

assuming $\mu_D = 4.5 \cdot 10^{-3} \text{ h}^{-1}$ in agreement with Flickinger and Drew (1999).

The accuracy of the experimental results under steady states conditions was assessed by the difference between the theoretical and the experimental uptake of lactose, as reported in § 2.1. In particular the stoichiometric consume of lactose F_L is expressed by eq. (T.2.4.1), and experimental consume is expressed as:

$$F_L^{\text{exp}} = D(L_0 - L) \quad (4.1)$$

Where L_0 and L are the molar concentrations of lactose in the feed medium and in the reactor, respectively. The relative error δ is expressed as:

$$\delta = \frac{F_L^{\text{exp}} - F_L}{F_L^{\text{exp}}} \quad (4.2)$$

Results

Figure 4.2 reports data measured during a typical continuous test carried out in the CSTR equipped with MF at pH=4.2. At the beginning of the test, the reactor was operated under continuous conditions with respect to the liquid ($D=0.028 \text{ h}^{-1}$) and batchwise with respect to the biomass ($d_1=0 \text{ h}^{-1}$). The operating conditions favored the accumulation of the biomass while keeping the metabolites concentration low reducing any inhibition effects. As the lactose concentration in the reactor approached 5 g/L ($t \sim 50 \text{ h}$), the biomass drawing stream (d_1) was set at about 10% of D : lactose conversion and solvent concentration decreased and approached steady state conditions at $t \sim 150 \text{ h}$. The reactor was kept under steady state conditions for

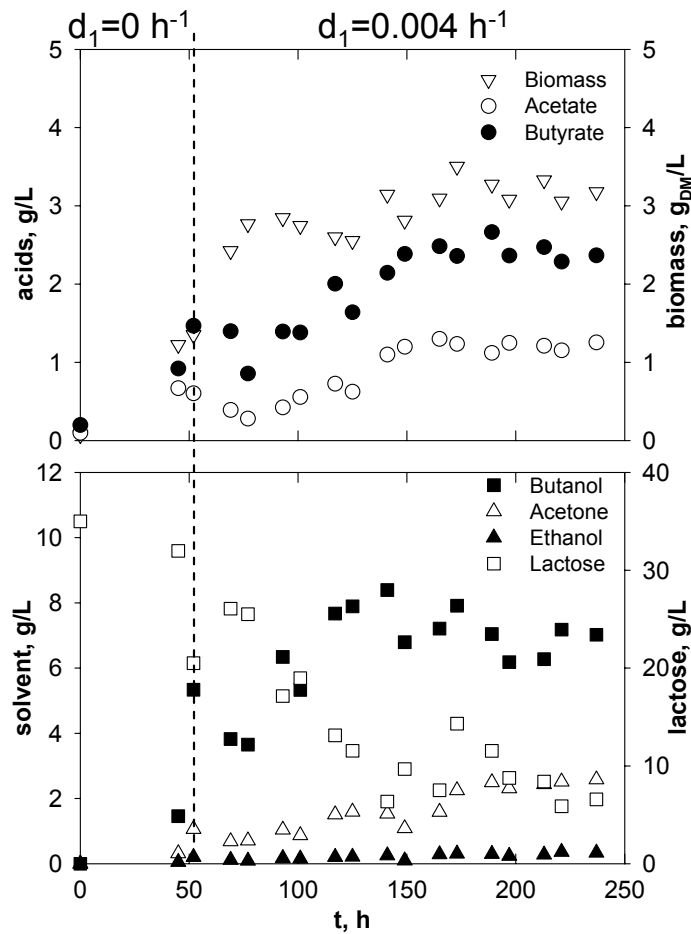


Figure 4.2. A typical continuous test carried out in the CSTR equipped with the microfiltration unit. $D=0.028 \text{ h}^{-1}$, $\text{pH}=4.2$, lactose in the feeding 47g/L.

about 100 h, more than 4 times the time-space. The steady state was characterized in terms of concentrations and productions of metabolites and cells, in agreement with Meyer and Papoutsakis (1989): butanol concentration 7 g/L, ratio B:Ac:Et about 35:10:1. Noteworthy, the low ethanol concentration supports the assumption adopted in § 2: negligibility of the ethanol production by acids uptake.

Table 4.2 reports the main results of a set of representative steady states investigated. The ethanol concentration was about one tenth of the other solvents. The largest butanol productivity was achieved at pH 4.2. The cells concentration decreased with the pH for value smaller than 4.2, in agreement with the model proposed in § 4.1.

Data measured under steady state conditions have been worked out in agreement with the set of equation reported in table 4.1 and results are reported in table 4.3. Analysing the table 4.3 some consideration may be derived and are reported hereafter.

- ❖ Acidogenic cells. The detrimental effects at pH lower than 4.2 are confirmed. In fact, the acidogenic cell concentration decreases markedly at pH=4.0 as it could be expected by the growth kinetics model reported in § 4.1.
- ❖ Solventogenic cells. A maximum appears at pH=4.2.
- ❖ Spore concentration. All steady states are characterized by a spore fraction larger than 40% and it is maximum at pH=4.0, very stressing conditions

Table 4.2. Main results of steady states in the CSTR equipped with the MF unit. Lactose concentration in the feeding: 47 g/L.

pH	4.0	4.2	4.5
$D \text{ (h}^{-1}\text{)}$	0.027	0.028	0.028
$d_1 \text{ (h}^{-1}\text{)}$	0.003	0.004	0.003
$X_T \text{ (g}_{DM}/\text{L)}$	2.76	3.16	3.22
$L \text{ (g/L)}$	25.5	8.7	10.8
Metabolite concentration (mg/L)			
AA	1270	1200	1260
BA	2810	2390	2590
Ac	1070	2090	2220
Et	234	273	346
B	2680	7110	5940
Metabolite molar flow rate (mM/h)			
F_{AA}	0.607	0.574	0.600
F_{BA}	0.915	0.778	0.842
F_{Ac}	0.530	1.031	1.095
F_{Et}	0.146	0.170	0.216
F_B	1.040	2.750	2.230
$\delta \text{ (%)}$	23	9	8

Table 4.3. Results of the growth model reported in tab. 4.1 applied to runs reported in tab. 4.2

pH	μ (§4.1 eq 8)	Y_{ATP} (§ 4.2 eq. 12)	f_1^{AA}	f_2^{BA}	f_4^{AA}	f_5^{BA}	f_6^B	μ_s	X_A	X_S	X_D
4.0	0.0035	0.3	0.8	1.2	0.2	0.3	0.6	0.0003	0.44	0.02	2.30
4.2	0.0234	1.9	1.0	1.4	0.4	0.7	1.9	0.0168	0.49	1.23	1.44
4.5	0.0149	1.2	1.0	1.5	0.4	0.7	1.4	0.0108	0.53	0.82	1.86

5 BIOFILM REACTOR

5.1 Butanol Production in a Continuous Packed Bed Reactor by *Clostridium acetobutylicum*

F. Napoli, G. Olivieri A. Marzocchella, M.E. Russo, P. Salatino
Dipartimento di Ingegneria Chimica
Facoltà di Scienze Biotechnologiche – Facoltà di Ingegneria
Università degli Studi di Napoli ‘Federico II’
P.le V. Tecchio, 80 – 80125 Napoli, Italy

Butanol production by immobilized *Clostridium acetobutylicum* in a Packed Bed Reactor (PBR) using Tygon® rings as carrier is analyzed. The medium was a solution of lactose (15-30 g/L) and yeast extract (3 g/L) to emulate cheese whey, a lactose-rich wastewater which is an abundant byproduct of the dairy industry. The reactor was operated under controlled conditions with respect to pH and to the dilution rate. The pH ranged between 4 and 5, the dilution rate between 0.54 h⁻¹ and 2.4 h⁻¹ (2.5 times the maximum specific growth rate of free cells).

Butanol production was characterized in terms of concentration of free cells and metabolites. Results were worked out to assess the rate of ABE production. The yields of the carbon source in cells, acids and solvents were also assessed. The reactor was operated under continuous conditions for more than one month. The optimal performance of the reactor was recorded at dilution rate of 0.97 h⁻¹: the butanol productivity was 4.6 g/Lh and the selectivity of solvent to butanol was 88%_w.

Keywords: butanol, selectivity, biofilm, PBR, Tygon®, *Clostridium acetobutylicum*, lactose

INTRODUCTION

Bioconversion of industrial wastewaters to produce biofuels and bulk chemicals is currently experiencing renewed interest. The objective of these processes is threefold: i) to reduce the impact of anthropic operations on the environment; ii) to produce bulk-chemicals by eco-sustainable processes; iii) to diversify the fuels sources, so as to increase the security of the fuel supply chain. The development of processes using biocatalysts – either microorganisms or enzymes produced by them – is based on the recognition of the conversion potential of a wide spectrum of organic substrates, natural and synthetic, sometimes recalcitrant. One drawback of the biotechnological pathway is represented by the fact that bio-based processes are characterized by low specific conversion rates. As a consequence, large

reactor volumes are usually required for processes operated at high throughput. This feature is responsible for the increased concern for the development of industrial scale biotechnological processes based on novel concepts and characterized by large degree of intensification.

In this frame butanol is gaining much interest because of its many advantages with respect other solvents (Cascone, 2008). The Acetone-Butanol-Ethanol (ABE) fermentation route was extensively followed during the first half of the twentieth century, but was discontinued in the early 1960s due to unfavorable economics with respect to petrochemical products (Ezeji *et al.*, 2004). Despite the strong interest in butanol production by fermentation, there are several factors that affect the economics of bioprocesses: the cost of usual substrates (typically corn and molasses); the low butanol productivity; the high cost of product recovery.

Among the saccharolytic butyric acid-producing *clostridia*, there are a number of species capable of producing significant amounts of neutral solvents during the late stage of batch fermentation, provided that appropriate operating conditions are established (*C. saccharoperbutylacetonicum*, *C. acetobutylicum*, *C. beijerinckii*, *C. aurantibutyricum*). The proven ability of these strains to utilize a wide spectrum of carbohydrates (Flickinger and Drew, 1999) has stimulated research on the use of cheap renewable feedstocks. Along this line, the large carbohydrates content of some wastewater streams, e.g. effluents from dairy industries, makes such wastewaters potential low-cost feedstocks for butanol production.

Cheese whey has attracted interest as an alternative substrate for AB fermentation because of its disposal issues (high BOD and COD), lactose content (about 5%_w), and availability in many countries. Previous studies addressing AB fermentation using whey or lactose as carbon source (Welsh and Veliky, 1984; Ennis and Maddox, 1985; Linden *et al.*, 1986) have pointed out that these substrates are characterized by rather low overall reactor productivities when batch conversions are considered, of the order of 0.1 g/Lh. On other hand, the selectivity to butanol is larger than that typically recorded during the fermentation of conventional substrates, resulting in more favorable economics of butanol recovery.

Though *clostridia* have been shown to be effective biocatalysts for ABE fermentation, there is still a lack of information in the literature supporting scale up and demonstration at the industrial scale (Cascone, 2008). Only few attempts have been reported in the literature to operate the continuous fermentation by means of *clostridia* strains confined in the reactor by immobilization (Meyer and Papoutsakis, 1989; Huang *et al.*, 2004; Qureshi *et al.*, 2005; Tashiro *et al.*, 2005; Ezeji *et al.*, 2007). A variety of supports have been employed for biofilm development such as sand (Fox *et al.* 1990, Gorris *et al.*, 1989), granulated activated carbon (Khan *et al.*, 1981), anthracite coal (Khan *et al.*, 1982), plastics and various kinds of clays (Qureshi *et al.*, 2000).

The present study moves one further step toward the characterization of butanol production by *C. acetobutylicum*. In particular, the study is focused on the development of a biofilm reactor. A packed bed typology (PBR) has been investigated as an intermediate step towards the development of a fluidized bed biofilm reactor. A solution of lactose and yeast extract has been used as medium to mime cheese whey wastewater. The use of a surrogate wastewater, rather than the real one, made it possible to better tune operating conditions without the variability of the medium properties that is typical of wastewaters. Butanol production was characterized in terms of lactose and metabolites concentrations, solvents productivity and selectivity to butanol.

MATERIALS AND PROCEDURE

Microorganism and culture media

Clostridium acetobutylicum DSM 792 was supplied by DSMZ. Stock cultures were reactivated according to the DSMZ procedure. Reactivated cultures were stored at 4°C in the

stock medium made of 50 g/L D-Lactose culture medium supplemented with CaCO_3 (18 g/L).

The culture medium used consisted of Yeast Extract (YE) at 3 g/L and of D-Lactose at concentration ranging between 15 and 30 g/L. The medium was sterilized in autoclave.

Apparatus

Figure 1 shows a sketch of the apparatus used for lactose fermentation. It consists of a fixed bed reactor, liquid pumps, a process heater, a device for pH control and on-line diagnostics.

The glass-lined bioreactor (250 mL volume) is jacketed for heat exchange. The head of the reactor is equipped with ports for injecting gas streams, the inoculation and the sampling. Nitrogen is sparged at the reactor bottom through a perforated pipe to support the anaerobic conditions. Reactor isothermal conditions were ensured by means of the external heater.

The apparatus for pH control consisted of a pH-meter, a peristaltic pump, a vessel with NaOH 1M solution and a pH-controller. The pH probe was inserted into an external container of 10 mL capacity fed with the stream issuing from the bioreactor (Fig. 1). The fluid feeding line was kept clean from clogging by gas stream and NaOH solution.

The sterile medium was fed at the bottom of the reactor by means of a peristaltic pump. The reactor, filled with the medium and the granular carrier, was sterilized in autoclave for 20 min at 121°C. The gas stream was sterilized by filtration, while the medium was loaded into a stainless steel tank and sterilized at 100°C for 1.5 h flushing saturated vapor at 3 atm in the internal coil.

Table 1 reports the properties of the carriers investigated. The plastic rings had length 0.5 cm, ID 3.2mm and 1.5mm thickness.

Diagnostics

Analysis of culture samples withdrawn from the fermenter was carried out after centrifugation at 11,000 rpm for 10 min. The free cells were characterized in terms of biomass concentration. The liquid phase was characterized to measure lactose and metabolites concentration. Lactose concentration (L) was measured by means of an enzymatic kit (Biopharm®). Metabolites concentrations were measured by means of a gas-chromatograph, equipped with a FID and with a capillary column poraplot Q (25 m x 0.32mm), using external standards. In particular the concentration of acetic acid (AA), butyric acid (AB), acetone (Ac), ethanol (Et) and butanol (B) were measured.

The distribution and the morphology of the biofilm were characterized by Scanning Electron Microscopy (SEM). Particles sampled at the end of each run were repeatedly rinsed with 9 g/L NaCl solution pH 7 and subsequently incubated for 15h with 3.5%_v glutaraldehyde at 4°C. After rinsing, samples were dehydrated through extraction with ethanol aqueous solutions at concentration of ethanol increasing progressively from 15%_v up to 99.8%_v (Stewart et al., 1995). After water extraction, the sample was air dried at 60°C and covered with gold layer as required by the SEM procedure. The samples were scanned and photographed with a scanning electron microscope.

Operating conditions and procedure

1 mL of stock culture was transferred in a 15 mL screw-cap bottles containing 50 mL of culture media (15 g/L of lactose). The culture was incubated for 2 days under batch anaerobic sterile conditions, then 10 mL of active culture was inoculated in the reactor.

Tests aimed at the characterization of solids as potential biofilm carriers were carried out in PBR in which the working volume was fixed at 150 mL. Typically, after 12-24 h of batch culture the lactose-bearing stream was fed to the reactor at the preset dilution rate (D).

The lactose concentration in the feed stream was set at 15 g/L. Samples of carriers harvested at the end of the tests were observed at SEM to characterize the microbial biofilm.

Tests aimed at butanol production were carried out with the PBR operated at selected conditions. Each steady state was characterized in terms of metabolites and lactose concentration. The mass of biofilm in the reactor was assessed at the end of the run according to the following procedure:

- the reactor was rinsed with sterile water to remove lactose and metabolites;
- the carrier particles with the biomass were harvested and dried for 1 day – Jouan RC10-10, at temperature of 40° C;
- the dry mass of the biomass and carriers was weighted;
- the biofilm was chemically detached from the carriers by washing with NaOH 10M solution;
- the bare carrier was recovered, autoclaved and washed several times to remove completely the biomass, then dried and weighted. Biofilm removal was verified by observation of the cleaned supports at SEM;
- the dry mass of the biofilm in the reactor was assessed as difference between the weight of the biofilm-covered carriers and that of the bare carriers.

All tests were carried out at 35°C. The pH set-point was investigated in the range between 4.0 and 5.0.

Assuming that the feeding was aseptically and free of metabolites and that the gas stripping of metabolites is negligible, the metabolites concentration and lactose concentration measured during the steady state conditions were worked out to assess the following data:

lactose-to-“i-species” fractional yield coefficient ($Y_{i/L}$) (i standing for either acids or solvents)

$$\text{acids} \quad Y_{A/L} = \frac{D(AA^{OUT} + BA^{OUT})}{D(Latt_{IN} - Latt_{OUT})} \quad (1)$$

$$\text{solvents} \quad Y_{S/L} = \frac{D(B^{OUT} + Ac^{OUT} + Et^{OUT})}{D(Latt_{IN} - Latt_{OUT})} \quad (2)$$

solvent productivity

$$\text{butanol} \quad \phi^B = D(B^{OUT} - B^{IN}) \quad (3)$$

$$\text{acetone} \quad \phi^{Ac} = D(Ac^{OUT} - Ac^{IN}) \quad (4)$$

$$\text{ethanol} \quad \phi^{Et} = D(Et^{OUT} - Et^{IN}) \quad (5)$$

The butanol selectivity (Φ) was assessed as the ratio between the butanol production rate and the sum of the production rate of all solvents:

$$\Phi = \frac{D(B^{OUT})}{D(B^{OUT} + Ac^{OUT} + Et^{OUT})} \quad (6)$$

The meaning of the symbols is reported in the Nomenclature section.

RESULTS

Selection of the carrier

Table 1 reports selected results regarding the biofilm formation on the carriers tested. The performances of the carriers were assessed in terms of free cell growth and of biofilm formation after 1 week incubation. Except for the silica sand, the investigated carriers did not suppress the growth of free cell cultures. Biofilm was observed on all solids except silica sand

and on glass beads (Figure 2). The biofilm on Tygon® rings appeared thicker and more uniform than that observed on the other solids. It should be noted that Tygon®, Teflon and Kartell® are hydrophobic solids, a feature that promote cell adhesion and biofilm formation.

Based on the results of the immobilization tests, Tygon® was chosen as the best suited solid carrier for continuous lactose fermentation in the packed bed biofilm reactor. This choice was suggested by the consideration that Tygon® is stable and by the favorable combination of carrier density and size, which would be reflected by the good quality of fluidization of these particles in the event that the bioreactor should be of fluidized bed type.

Biofilm reactor start-up

Figure 3 shows the start up of the reactor loaded with 69 g of Tygon® rings. The concentration of metabolites and the pH are reported as a function of the time. The reactor was inoculated at $t=0$ and operated batchwise with respect to the liquid phase for 20 hours, a time interval sufficient for biofilm formation (Qureshi *et al.* 2005). Thereafter, the reactor was switched to continuous operation by steadily feeding the lactose-bearing medium (15 g/L). The dilution rate was set at $D=0.40\text{ h}^{-1}$ and the pH was gradually increased from 5.0 to 5.5 to force the fermentation under acidogenesis conditions. After about two days of incubation the carriers were covered by a light layer of biofilm visible to the direct observation and the dilution rate was increased to promote biofilm production with respect to free cell growth. At $D=0.80\text{ h}^{-1}$ - a value close to the maximum specific growth rate of free cells under the operating conditions adopted (Napoli *et al.*, 2009) - the lactose concentration in the reactor decreases at a rate larger than the parallel increase of metabolites content, probably as a consequence of biofilm growth.

The dilution rate was still increased at $t=140\text{ h}$ to compensate for the gradual lactose depletion ($L<2\text{ g/L}$). In particular, D was set at 2.4 h^{-1} , about 2.5 times the maximum specific growth rate. Under the selected conditions the biofilm reactor approached a steady state regime since $t=190\text{ h}$. All together, the biofilm reactor start up took about 9 days, during which biofilm growth was extensive.

Butanol production

Once a substantial amount of biofilm was formed, at $t=216\text{ h}$ the bioreactor operating conditions were adjusted so as to promote butanol production: pH at 4.0 and D at 0.54 h^{-1} . The value of pH was set in agreement with previous investigation carried out in batch reactor (Napoli *et al.*, 2009): cells shift to the solventogenesis phase at $\text{pH}=4.0$. Figure 4 reports the concentration of lactose and metabolites measured in the reactor for a continuous culture operated at $D=0.54$. As expected the solvents were continuously produced, besides the acids, confirming the co-existence of biomass production with production of butanol and acetone. However, lactose conversion and solvents progressively decreased consistently with a progressive extinction of the fermentation process. Lactose conversion and solvent production was immediately recovered as pH was increased up to 4.3 at $t=287\text{ h}$. Steady state conditions were approached in about two days and lasted for about 6 days (about 60 times the reactor space-time).

Table 2 reports selected data regarding the steady state characterized by $D=0.54\text{ h}^{-1}$ and $\text{pH}=4.3$. The reactor performance was characterized in terms of lactose and metabolites concentration, lactose conversion degree, acids and solvents yield, solvents productivity and butanol to solvent selectivity. Notwithstanding the operating conditions adopted promoted the solvents production, the acids production (1.43 g/Lh) was still remarkable with respect to the solvent production (0.77 g/Lh)

At $t=472\text{ h}$ the operating conditions were changed in order to increase the solvents production (Fig. 4). The lactose concentration in the feed was set at 30 g/L , closer to values typical of cheese whey and pH was further increased to 5.0. After about four days of adaptation to the new operating conditions, the dilution rate was set at 0.97 h^{-1} , in agreement

with previous investigation that pointed out high solvent productivities at values of $D \approx 1 \text{ h}^{-1}$ (Qureshi and Maddox, 1995; Huang *et al.*, 2004; Zhang *et al.*, 2009). The biofilm PBR approached a new steady state conditions ($t \approx 616 \text{ h}$) and it was successfully operated for further 134 h (about 140 times the reactor space-time).

As operation of the biofilm PBR was stopped, biomass concentration turned out to be $74 \text{ g}_{\text{DM}}/\text{L}$, corresponding to a biomass-to-carrier ratio of $0.16 \text{ g}_{\text{DM}}/\text{g}$. Biomass concentration is larger than the value reported by Qureshi *et al.* (2005), who used bone char as biofilm carrier ($0.087 \text{ g}_{\text{DM}}/\text{g}$). It is concluded that Tygon[®] is a very effective support for immobilization of *Clostridia* cells and butanol production.

Selected data corresponding to the test whose data are shown in fig. 5 have been reported in table 2. As a general trend, the reactor performance improved with D and/or pH .

The analysis of the effect of the operating conditions on reactor performance are now in order. The attention is focused on the effects on butanol selectivity and productivity.

The average solvents concentration and the butanol/acetone mass ratio (R) increase as pH and/or D increase. In particular R was 6 at $\text{pH}=4.3$ and 88 at $\text{pH}=5.0$. The ratio R recorded at the highest pH is even larger than that estimated by Linden *et al.* (1986) during whey fermentation, ranging between 12:1 and 20:1. The butanol/acetone ratio is larger than that typically observed during glucose fermentation (3:1) (Jones and Woods, 1986). The lower concentration of acetone at higher pH/D is in agreement with the lower concentration of acids, taking into account that acetone production implies acids reconversion.

Butanol selectivity was larger than $80\%_{\text{w}}$ for the steady states investigated, as large as 88% at the largest value of D . This result is promising in the light of the possible advantage in the butanol recovery and concentration stage (Ezeji *et al.*, 2004; Papoutsakis, 2008). Indeed, higher butanol concentration coupled with high selectivity decrease the cost of the distillation train, which represents the typical downstream processing method (Liu and Fan, 2004; Oudshoorn *et al.*, 2009).

The analysis of the acids and solvent yields confirms the previous scenario. Acids yield is 2-3 times the solvent yield at low pH or D . At higher pH or D the comparison of the yields is more favorable to the solvent, supporting the marked shift of the lactose conversion towards the direct pathway.

The productivity of solvents estimated during the steady states deserves consideration. At $D=0.97 \text{ h}^{-1}$ and $\text{pH}=5$ the solvents productivity was about 5.0 g/Lh , six times the value obtained at low pH and dilution rate. The maximum observed productivity was still larger than that reported by Qureshi and Maddox (1987). They measured a solvents productivity of 4.5 g/Lh for lactose fermentation carried out under similar operating conditions and using bonechar as carrier.

Altogether, the experimental results suggest that the performance of the biofilm reactor improves with pH . The role of pH is consistent with results reported for a continuous biofilm reactor (Huang *et al.*, 2004). However, results are apparently at odds with data reported by Jones and Woods (1986) for batch and continuous culture of free cells. In particular, for free cells the decrease of pH improves the solventogenesis phase. The apparent contradiction may be reconciled by taking into account the role of transport phenomena in the biofilm. The onset of significant nonuniformities of pH and substrates and metabolites across the biofilm (Qureshi *et al.*, 2005) justifies the higher optimal pH found for the biofilm reactor: setting $\text{pH}=4$ in the bulk, the inner region of the biofilm would experience pH lower than 4 that would bring about a reduction of cell activity.

CONCLUSIONS

The continuous conversion of lactose to Acetone-Butanol-Ethanol by *Clostridium acetobutylicum* was successfully accomplished in an anaerobic biofilm packed bed reactor.

Several biofilm carriers were characterized and Tygon® rings were eventually selected as optimal. The reactor was successfully operated for more than 1 month under operating conditions ranging in broad intervals of pH (4.0-5.1), dilution rate (0.54-1.0 h⁻¹) and lactose concentration in the feeding (15-30 g/L). Several reactor steady states were characterized in terms of solvents productivity and yields and of butanol selectivity.

The tests suggest that the reactor performances improve with D and pH within the intervals investigated. The maximum lactose-to-butanol productivity (4.43 g/Lh) and butanol selectivity to solvents (88%_w) were among the largest values reported in the literature. Moreover, the results highlighted the effectiveness of Tygon® as a potential biofilm carrier for butanol production.

Results regarding the effects of pH on the reactor performance suggest a likely influence of transport phenomena across the biofilm. As pH typically decreases from the surface to the inner part of the biofilm, an optimal value of pH in the bulk of the culture higher than that applicable to free cells processes has to be established.

Acknowledgments

The Authors are indebted with Mrs Sabrina Manzi for her assistance in experimental investigation.

NOMENCLATURE

AA, Ac, B, BA, Et, L acetic acid, acetone, butanol, butyric acid, ethanol, lactose

D dilution rate, 1/h

Y_i fractional yield

P productivity

Greek Letters

μ specific growth rate

Φ selectivity

Subscripts

AA, Ac, B, BA, Et, L acetic acid, acetone, butanol, butyric acid, ethanol, lactose

A total acids

S total solvents

BIBLIOGRAPHY

- Annachatre, A.P., Bhamidimarri S.M.R., "Microbial attachment and growth in fixed-film reactors: process startup considerations", *Biotech. Adv.*, 1992, vol. 10, 69-91.
- Cascone, R. (2008). Biobutanol – "A Replacement for Bioethanol?", *Chem. Eng. Prog.*, August, S4-S9.
- Ennis, B. M., I. S. Maddox, "Use of *Clostridium acetobutylicum* P262 for production of solvents from whey permeate", *Biotechnol. Lett.*, 1985, vol. 7, 601-606.
- Ezeji, T.C., N. Qureshi and H.P. Blaschek, "Bioproduction of butanol from biomass: from genes to bioreactors", *Curr. Op. Biotechnol.*, 2007, vol. 18, 220-227
- Ezeji, T.C., Qureshi, N., Blaschek, H.P. "Butanol fermentation research: upstream and downstream manipulations". *The Chemical Record*, 2004, vol 4, 305-314.
- Flickinger, M. C., S.W. Drew, "Bioprocess technology: fermentation, biocatalysis, and bioseparation", Wiley-Blackwell, John Wiley & Sons Inc., 1999, vol 1 – 5.
- Fox P., Mt Suidan, J.T. Bandy, "A comparison of media types in acetate fed expanded-bed anaerobic reactors". *Water Research*, 1990, vol.24. 827-835.
- Gorris LGM, JMA Van Deursen, C. Van Der Drift, GD Vogeles, "Biofilm development in laboratory methanogenic fluidized bed reactors", *Biotech. Bioeng.*, 1989, vol. 33, 687-693.

- Huang WC, Ramey DE, Yang ST: "Continuous production of butanol by *Clostridium acetobutylicum* immobilized in a fibrous bed bioreactor" Appl. Biochem. Biotechnol., 2004, vol. 113 887-898.
- Jones, D.T., D.R. Woods, "Acetone-Butanol Fermentation Revisited", Microb. Rev., 1986, Dec, 484-524.
- Khan KA, MT Suidan, WH Cross, "Anaerobic Activated Carbon Filter For The Treatment Of Phenol-Bearing Wastewater" J. Water Pollut. Control. Fed., 1981, vol 51 1519-1528.
- Khan KA, MT Suidan, WH Cross, "Role of surface active media in anaerobic filters", J. Envir. Eng., 1982, vol. 108, 269-285.
- Linden, J. C., A. R. Moreira, T. G. Lenz, "Acetone and Butanol", p. 915-931. In C. L. Cooney and A. E. Humphrey (ed.), Comprehensive biotechnology. The principles of biotechnology: engineering consideration, 1986, Pergamon Press, Oxford.
- Liu J. and L.T. Fan., "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, 2004; vol. 20, 1518-1527.
- Meyer, C.L. and E.T. Papoutsakis, "Continuous and biomass recycle fermentations of *Clostridium acetobutylicum*. Part 1: ATP supply and demand determines product selectivity", Bioprocess Engineering, 1989, vol. 4, 1-10
- Napoli, F., Olivieri G., Marzocchella A., Salatino P., "An Assessment of the Kinetics of Butanol Production by *Clostridium acetobutylicum*". Int. J. Chemical Reactor Engineering, 2009, vol.7, A45
- Oudshoorn A., L.A.M. Van der Wielen, A.J.J. Straathof, "Assessment of Options for Selective 1-Butanol Recovery from Aqueous Solution" Ind. Eng. Chem. Res. 2009.
- Papoutsakis E.T., "Engineering solventogenic clostridia", Current Opinion In Biotechnology, 2008, vol. 19, 420-429.
- Qureshi N, B A Annous, T C Ezeji, P Karcher and I S Maddox, "Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates", Microbial Cell Factories, 2005, vol. 4, 24
- Qureshi N., Schripsema J., Lienhardt J., Blaschek H.P., "Continuous solvent production by *Clostridium beijerinckii* BA101 immobilized by adsorption onto brick", World J. Microbiol. Biotechnol., 2000, vol. 16, 377-382
- Qureshi, N., I.S. Maddox, "Continuous production of acetone-butanol-ethanol using immobilized cells of *clostridium acetobutylicum* and integration with product removal by liquid-liquid extraction", Journaol of Fermentatioan and Bioengineering, 1995, vol. 80, 185-189.
- Qureshi N., Maddox I.S., "Continuous solvent production from whey permeate using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar, Enz Microbial Technol 1987, vol. 9, 668-67
- Stewart P., Murga S., et al., "Biofilm structural heterogeneity visualized by three microscopic methods", Water Research, 1995, vol. 29, 2006-9.
- Tashiro, Y., K. Takeda, G. Kobayashi and K. Sonomoto, "High production of acetone-butanol-ethanol with high cell density culture by cell-recycling and bleeding", J. Biotechnol., 2005, vol. 120, 197-206
- Tyagi R.D., Ghose T.K: "Studies on immobilized *Saccharomyces cerevisiae*. I. Analysis of continuous rapid ethanol fermentation in immobilized cell reactor" Biotechnol. Bioeng., 1982, vol. 24, 781-795.
- Welsh, F. W., I. A. Veliky, "Production of acetone butanol from acid whey", Biotechnol. Lett., 1984, vol. 6, 61-64.

Zhang Y., Y.Yujiu Ma, F. Yang, C. Zhang, “Continuous acetone–butanol–ethanol production by corn stalk immobilized cells”, J. Ind. Microbiol. Biotechnol., 2009, vol. 36, 1117–1121.

Table 1. Properties of the carriers tested and their performance during fermentation.

Carrier	Diameter (μm)	Density (kg/m ³)		Free cell growth	Biofilm growth
Silica gel	600	720	Microporous	+	+
Pumic	500	1050	Macroporous	+	+/-
Glass beads	400	2540	Impervious	+	-
Silica sand	600	2600	Impervious	-	+
Tygon®	3800-6000	1180	Impervious	+	+
Teflon	3000-5000	2200	Impervious	+	+
Kartell®	4000-6000	1300	Impervious	+	+

Table 2. Operating conditions and selected variables recorded during steady cultures of *C. acetobutylicum* in PBR.

Operating conditions			
	D [h ⁻¹]	0.54	0.97
	pH	4.34	5.08
	Lactose in the feed [g/L]	15.0	30.0
Results			
	Metabolites [g/L]		
	Ethanol	0.05	0.55
	Acetone	0.18	0.05
	Butanol	1.20	4.59
	Acetic acid	0.53	0.67
	Butyric acid	2.12	1.48
	Lactose [g/L]	6.3	11.8
	Solvent Productivity [g/Lh]		
	Ethanol	0.03	0.53
	Acetone	0.10	0.05
	Butanol	0.65	4.43
	Butanol selectivity[g/g]	0.83	0.88
	Solvents yield [g/g]	0.15	0.28
	Acids yield [g/g]	0.28	0.12
	Lactose consumed [g/L]	9.6	18.5

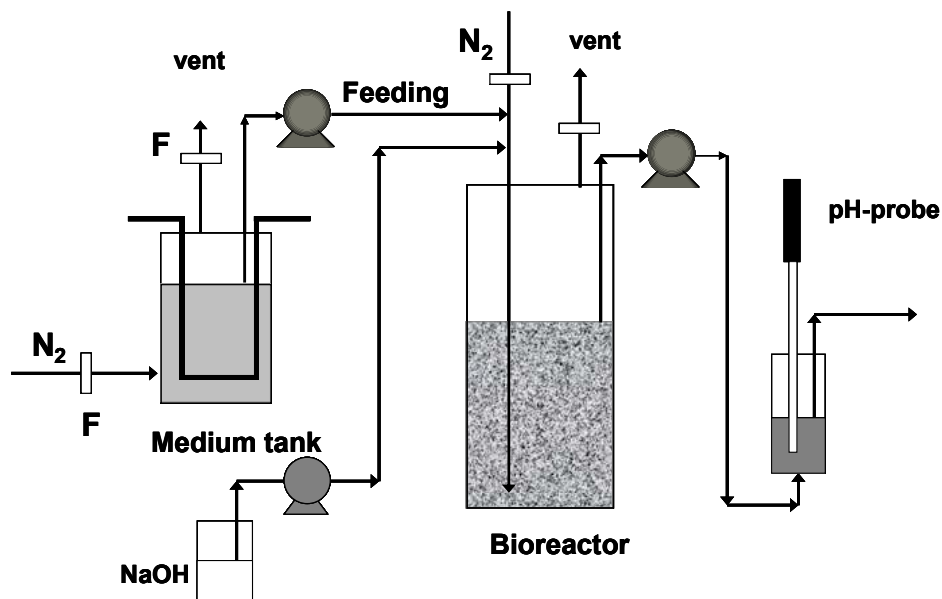


Figure 3 The experimental setup used in continuous tests embodying the packed bed biofilm reactor . F) gas sterilization filter

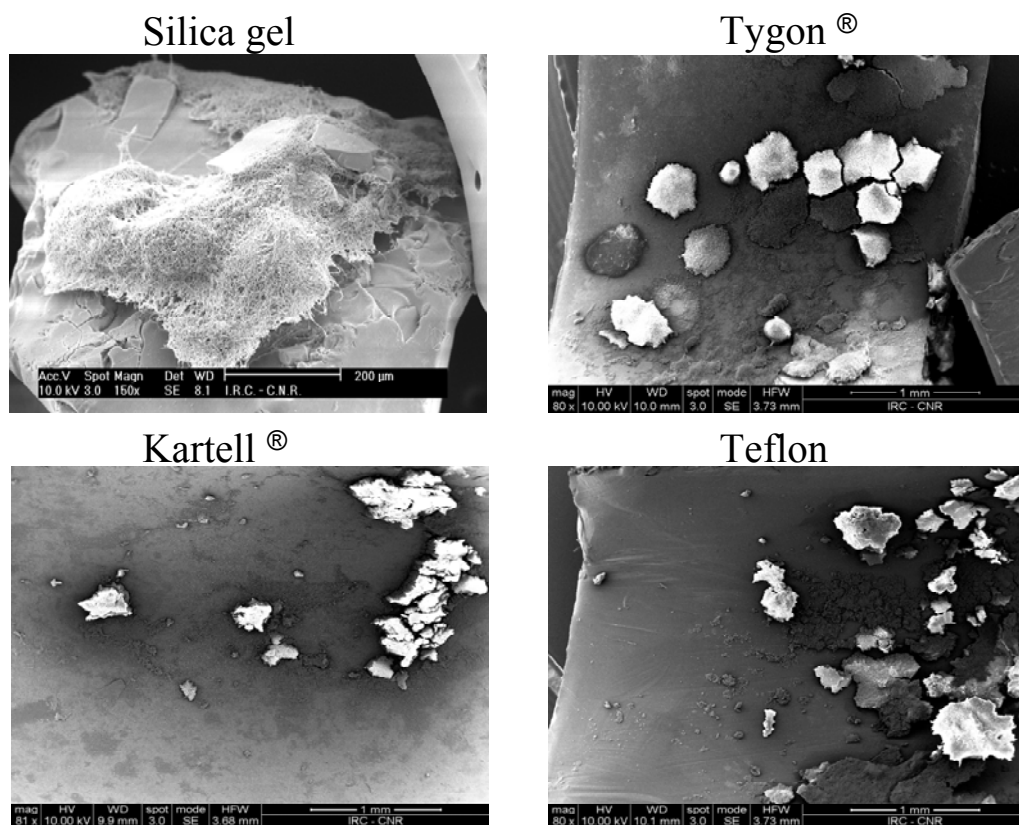


Figure 4 SEM micrographs of biofilm-covered carriers. Particles sampled after 1 week incubation. PBR operated at $D=0.5 \text{ h}^{-1}$. Lactose concentration in the feeding: 15 g/L.

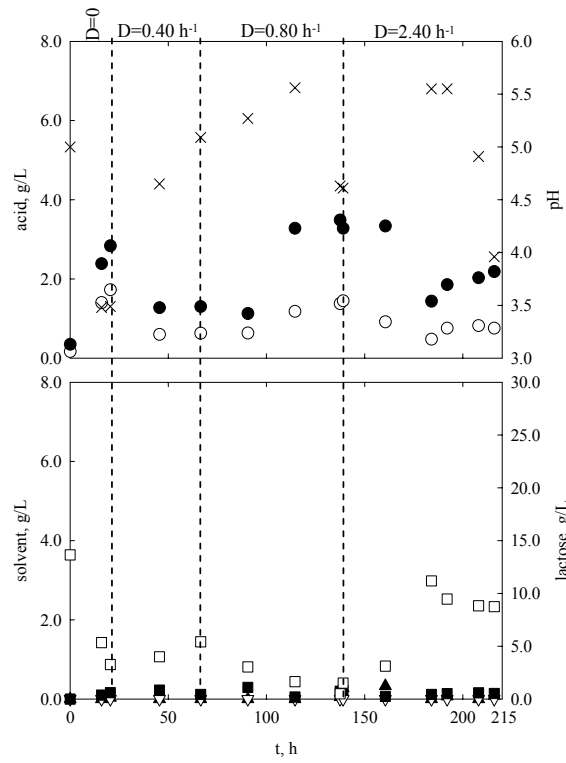


Figure 5 Variables recorded during PBR start up. Lactose concentration in the feeding: 15 g/L. × pH, ● acetic acid, ○ butyric acid, □ lactose, ■ butanol, ▽ acetone, ▲ ethanol.

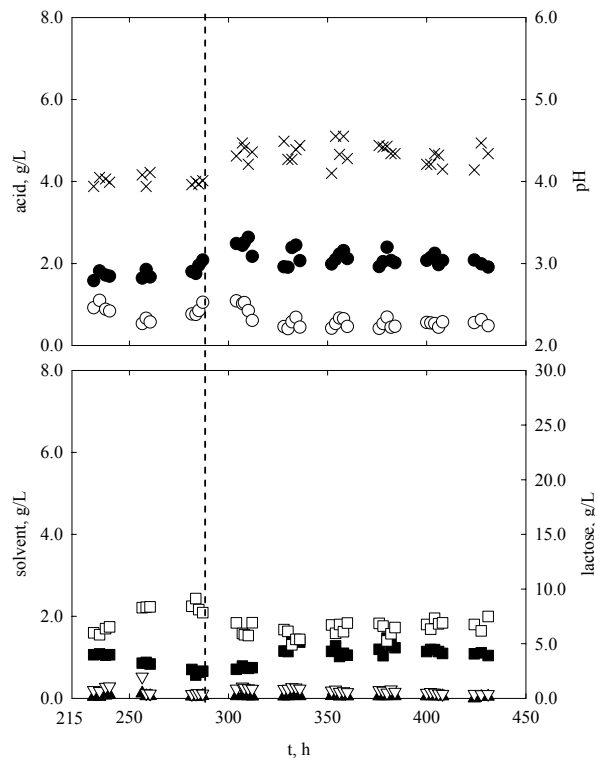


Figure 6 Variables recorded during steady operation of the PBR: $D=0.54 \text{ h}^{-1}$; lactose concentration in the feeding: 15 g/L. Key to symbols in fig. 3. The vertical line marks the pH increase.

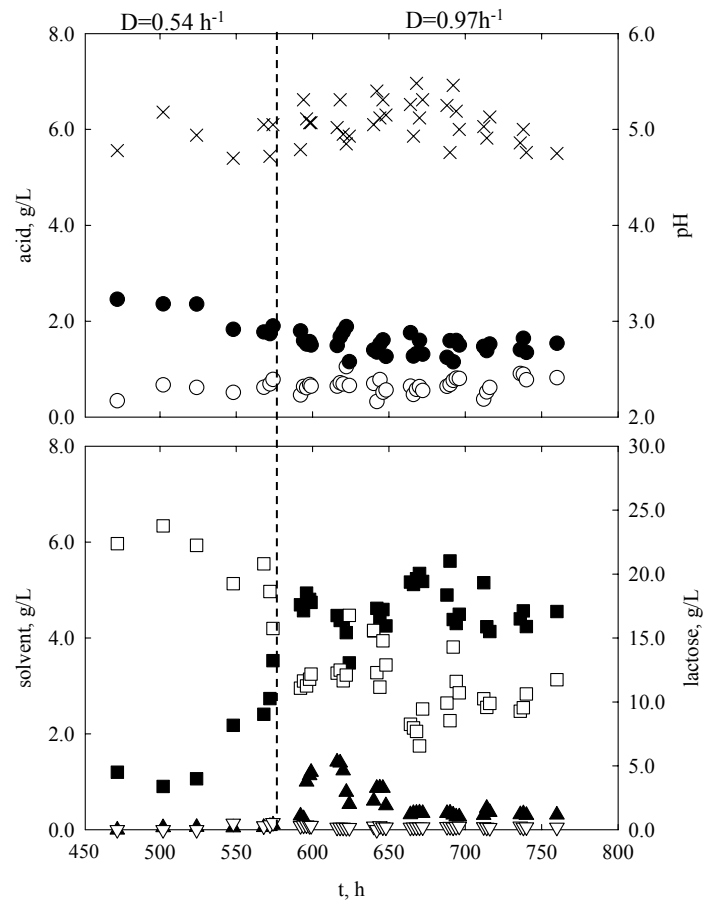


Figure 7 The approach to the steady state of PBR. Dilution rate= 0.97 h^{-1} ; Lactose concentration in the feeding= 30 g/L ; pH=5.0 Key to symbols in fig. 3.

6. ECONOMIC EVALUATION

6.1 Optimization of Solvent Recovery in the Production of Butanol by Fermentation

F. Napoli, A. Marzocchella, P. Salatino

Dipartimento di Ingegneria Chimica - Università degli Studi di Napoli "FEDERICO II"
P. V. Tecchio 80 - 80125 Napoli, Italy

The current energetic scenario has revived the interest for Acetone-Butanol-Ethanol (ABE) fermentations. An open issue is represented by the recovery process of ABE from the fermentation broth, which can be extremely expensive due to the low concentration of products that establish during the fermentation. Since the ABE production kinetics is characterized by product inhibition, the ABE concentration in the bioreactor is typically smaller than 20g/L. The present work reports on the assessment of a cost-effective flow-sheet for the recovery of butanol produced by fermentation. The study is based on approximated cost-estimation methods integrated with the simulation software Aspen Plus®. The recovery line investigated includes gas-stripping, absorption of butanol in a selected liquid phase, distillation to separate the butanol from the selected liquid. The design variables are selected so as to correspond to the degrees of freedom of the process. A preliminary determination of plausible values of the venture profit of the process has been attempted as a function of operating conditions.

Keywords:

ABE process, butanol, economic optimization, Venture profit, downstream processing

INTRODUCTION

The current energetic scenario has revived the interest in Acetone-Butanol-Ethanol (ABE) fermentations. Recent developments in molecular techniques applied to solventogenic microorganisms in combination with recent advances in fermentation systems and downstream processing have contributed to improve ABE fermentation processes feasibility and competitiveness (Cascone, 2008). The challenges raised over the last years as regards ABE production may be summarized in: i) use of renewable resources as substrates; ii) selection of strains (e.g. *Clostridia*) characterized by high ABE productivity; iii) development of new fermentation systems; iv) development of new downstream strategies for enhanced solvent recovery. The selection of unconventional substrates is favoured by the ability of *Clostridia* strains to metabolize a wide range of carbohydrates like glucose, lactose, etc..., that are typically present in wastewater streams e.g. from the food industry. In particular, studies available in literature point out the potential of the ABE production process by fermentation of lactose or cheese whey (Welsh and Veliky, 1986).

A drawback to the ABE production by fermentation is the expensiveness of downstream processing aimed at isolating and purifying the products. The key aspects responsible for the expensiveness of recovery are: i) the small concentration of solvents in the fermentation broth (20 g/L), that are even smaller when cheese whey is used as feedstock (13-18 g/L); ii) the unfavourable thermodynamics of the butanol-water system (the boiling point of butanol is higher than that of water, 118°C at 1 bar); iii) the possible occurrence of unconverted carbohydrates in fermentation broth which may clog recovery apparatus when high temperature processes are adopted. As a consequence, conventional technologies for butanol recovery, e.g. distillation trains, result not competitive and suitable combinations of conventional unit operations - gas stripping, absorption, liquid-liquid extraction, ... - are

suggested to make the recovery processes economically feasible (Liu and Fan, 2004).

The present study reports preliminary results of a study aiming at investigating the economical feasibility of butanol recovery from ABE fermentation by means of an unconventional flowsheet. The reference background is represented by the production of solvents by anaerobic fermentation using cheese whey as the substrate. The basic operations of the proposed flowsheet were: gas stripping, absorption, distillation. The flowsheet has been simulated by means of the Aspen Plus® process simulator and optimized assuming the venture profit as objective function.

PROCESS DESCRIPTION

The cheese whey - a by-product of cheese manufacturing – may be adopted as a convenient fermentation substrate. In fact, lactose (4 – 5 %_w) and proteins and fat (<1.5%_w) are a sufficient medium for clostridia fermentation. Table 1 reports the typical composition of the fermentation broth at the end of the bioconversion. The butanol concentration is too low to make distillation an economically competitive recovery process.

The butanol manufacturing process may be split in three section: in upstream processing, in bioconversion of the feedstock and the downstream processing. The recent progress in fermentation process (biofilm reactor) and the use hyper-butanol-producing culture, characterized by high butanol productivity and high solvent tolerance, permit continuous production using single or double stage at relative high dilution rate (Qureshi, 2000),

Cheese whey, a byproduct of the cheese manufacturing industry, can be used like substrate without very expansive upstream process (Qureshi, 2005). so analysis of the upstream unit, bioreactor and of ancillary apparatus associated with the bioconversion process were not taken into account in the present study. The attention was focused on recovery of butanol from a solution at 35°C, atmospheric pressure and characterized by the composition reported in table 1.

Butanol recovery flowsheet

The main steps of the downstream process to recovery butanol are: gas stripping, absorption and distillation. The flowsheet also includes mixers, a condenser, heat exchangers and decanters. The main advantage of the proposed flowsheet are: possible operation of the gas stripping stage directly in the fermentation unit; operation of butanol distillation section with a butanol feeding of orders of magnitude larger than that reported in table 1. The first aspect is attractive as it may contribute to enhance the potential of the fermentation unit. In fact, continuous subtraction of the solvents from the culture may improve the process due to the product-inhibited character of the relevant processes.

The main features of each unit are hereby described.

Gas stripping unit. A gas stream, air or inert, using gas self-produced by fermentation, is used to strip volatile compounds (water, butanol, acetone and ethanol) from the fermentation broth. Low volatile components (acids, lactose, proteins and fats), free cells, and the sizable fraction of water are withdrawn from the gas stripper unit and delivered to a wastewater treatment system. The separation is strongly favoured by the very low volatility even with respect to the possible azeotrope ethanol-water and water-butanol. The design variables adopted for this unit are: the number of theoretical plates (N_{strip}), the gas flow rate fed to the unit (G), and the temperature of the feed stream (T).

The gas stream from the stripping unit is delivered to a condenser. The liquid streams are mixed with the fermentation broth or sent to the distillation train depending on the composition (the water-butanol system is characterized by an immiscibility range).

Gas absorbing unit. The absorber is dedicated to the recovery of butanol from the gas stream. The liquid phase should be characterized by high affinity towards the butanol and low affinity towards the water. Eicosanol (X) fulfils the recalled prerequisites (table 2) and has been adopted as liquid solvent [3].

This unit removes most of the butanol from gas phase. The components of the liquid stream from the bottom of the absorber tower are eicosanol, butanol and water. The design variables selected for this unit are: the number of theoretical plates (N_{abs}), and the flow rate of the liquid solvent (X_{flow}).

Distillation units. A distillation train is dedicated to recover butanol from the water-eicosanol solution and to recycle water-butanol azeotropic solution and eicosanol. The overhead product from the first unit – butanol-water solution at the azeotropic composition (75% of water on molar basis) – is recycled just after the gas stripping unit, and the bottom product - butanol-eicosanol solution - is fed to the second unit. The purity of the second unit product streams have been set at high values: butanol at 0.995 (on molar basis) and eicosanol at 0.999. Despite the strong separation efficiency request in the second distillation unit, the size of this unit is not very large because the system butanol-eicosanol is characterized by large relative volatility. The design variables of the distillation train are: the number of theoretical plates of the first unit (N_{1dist}), the reflux ratio of the first unit (R_1) and the second unit (R_2), the distillate flow rate of the first unit (D_1).

The flowsheet reported in Fig. 1 has been simulated and sized by means of Aspen Plus®.

COST ESTIMATION

The cost estimation for the conceptual design of the flowsheet dedicated to the recovery of butanol from the ABE stream has been carried out following the procedure proposed by Happel and Jordan (1975). In particular, the total capital requirement (I, \$) has been estimated considering the purchased cost of the main units and adopting the factorial method for ancillary apparatus and installation costs. Purchased cost for the main units of the flowsheet have been estimated in agreement with correlations reported in Peters et al. (2003).

The venture profit (V, \$/year) of the process has been adopted as objective function to be maximized as a function of the nine degrees of freedom of the system. A compact form of the venture profit is:

$$V = S - C - (S - C - Id)t - eI - iI \quad (1)$$

where S is the net income from the annual sales [\$/yr], C the annul manufacturing costs [\$/yr], t the income tax rate (\$/\$), d (1/yr) the yearly fractional depreciation rate of value of the fixed equipment allowed by tax authorities, i (1/yr) the rate of return of the firm, and e (1/yr) the yearly fractional depreciation rate [5]. Tentative reference values of $t=0.5\$/\$,$ expected project life $n=10$ (accordingly $d=0.1$ 1/yr for straight line depreciation), $i=0.07$ 1/yr and $e=0.069$ 1/yr (depreciation according to the sinking fund method) have been used for this preliminary economic assessment (Peters et al. 2003).

RESULTS

The simulations have been carried out on the basis of a processed stream of flow rate $F=5 \cdot 10^5$ tons/year. The maximum venture profit is $1.4 \cdot 10^6$ \$/year with a butanol recovery of 0.996 and a total income from sales of $4.6 \cdot 10^6$ \$. The optimum conditions is characterized by: i) gas stripping unit equipped with theoretical plates $N_{strip}=6$, feed temperature $T=50^\circ\text{C}$ and gas flow rate $G=1000$ kmol/h; ii) absorber unit equipped with theoretical plates $N_{abs}=5$ and operated with a molar flow rate of extracting stream $X_{flow}=40$ kmol/h; iii) the first distillation

unit equipped with $N_{1\text{dist}}=10$ theoretical plates, operated at reflux ratio $R_1=0.05$ and a distillate flow rate $D_1 = 14$ kmol/h; iv) the second distillation unit operated at reflux rate $R_2=0.75$.

Table 3 reports the main results of the cost estimation under the optimized conditions. It results that the distillation train is still the most relevant item in terms of equipment cost, 51% of total, despite the advantageous operating conditions of these units. Assuming the economic scenario reported above (i, e, d, t) it results that the contribution from depreciation to the venture profit is about $1.3 \cdot 10^5$ \$/yr, that is about one tenth of the manufacturing cost.

Figure 2 shows the venture profit, normalized with respect to the maximum value, as a function of the design variables. The sharpness of the maximum is to be associated to the sensitivity of the venture profit with respect to the selected design variable. Data show that the venture profit increases with N_{strip} and X_{flow} , to become nearly constant for values larger than 4 and 12 ton/h, respectively. The decrease of the venture profit for N_{strip} smaller than 4 is due to the decrease of the gas stripping efficiency. On the other hand, data reported in fig 2B and 2C show that the venture profit is very sensitive on the feed temperature T and on the and gas flow rate fed to the stripping unit. Sensitivity of the venture profit to the other project variables results less than 10% (data not reported).

Figure 3 reports results of the analysis as regards the effect of the flow rate of the processed stream - composition reported in table 1 - on venture profit, total investment and labour cost. The design variables were set at the value corresponding to the optimal conditions estimated for the base case ($5 \cdot 10^5$ ton/yr). It results that the downstream process is profitable for feed flow rates exceeding 800 kmol/h ($1.1 \cdot 10^5$ ton/yr).

CONCLUSIONS

A tentative design of a solvent recovery section for butanol production by fermentation from an arbitrary feedstock is presented. Proper selection of gas stripping, absorption and distillation units enables recovery of a high-purity (99.5% mol/mol) of butanol from relatively dilute ($<0.5\%$ mol/mol in butanol) fermentation broth. A preliminary assessment of the economics of the recovery process has been carried out with reference to a tentative economic scenario, suggesting that the process may be feasible provided that the throughput of the section exceeds a given threshold (around $1 \cdot 10^5$ ton/yr in the simulated case). The low concentration of Acetone and ethanol make their distillation recovery very expensive, emphasizing the necessity to enhance the process selectivity.

REFERENCES

- Cascone R. Biobutanol - A Replacement for Bioethanol?. Chem Eng Prog, 2008; S4-S9
- Ezeji TC, Qureschi N, Blaschek HP. Butanol Fermentation Research: Upstream and Downstream Manipulations. The Chemical Record, 2004; 4 (5):305-314.
- Gapes JR. The Economics of Acetone-Butanol Fermentation: Theoretical and Market Considerations. J. Mol. Microbiol. Biotechnol, 2000; 2 (1):27-32.
- Happel J and Jordan DG. Chemical Process Economics, Dekker Inc., New York, 1995
- Humphreys K and Lloyd M. Project and cost engineer's handbook (3rd edition), New York: M. Dekker, 1993
- Jones D.T. and D.R. Woods. Acetone-Butanol Fermentation Revisited. Microb. Rev., 1986: 484-524
- King C. Separation Processes(2nd edition), Chemical Engineering Series, MacGrawhill, 1987
- Lenz TG and Moreira AR. Economic evaluation of the Acetone-Butanol Fermentation. Ind. Eng. Chem. Prod. Res. Dev., 1980; 19:478-483
- Liu J. and L.T. Fan. 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, 2004; 20: 1518-1527

- McCabe, Smith, Harriott. Unit Operations of Chemical Engineering (5th edition), McGrawhill, 1993.
- Peters MS, Timmerhaus K and West RE. Plant Design and Economics of Chemical Engineers, McGraw-Hill, New York, 2003
- Qureshi N, Annous AB. Biofilm reactors for Industrial Bioconversion Processes: Employing Potential of Enhanced Reaction Rates. Microbial Cell Factories, 2005; 4:24
- Qureshi N, Schripsema J. Continuous solvent production by *Clostridium beijerinckii* BA101 immobilized by adsorption onto brick. World Journal of Microbiology & Biotechnology, 2000; 16:377-382
- Qureshi N, Maddox IS. Continuous solvent production from whey permeate using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. Enz Microbial. Technol., 1987; 9:668-671.
- Qureshi N, Blaschek HP. Evaluation of recent advances in butanol fermentation, upstream, and downstream processing. Bioproc Biosys Eng, 2001; 24:219-226
- Sobocan G and Glavic P. Optimization of ethanol fermentation process design. Appl. Therm. Eng., 2000; 20(6):529–543.
- Welsh FW and Veliky I. Production of acetone-butanol from acid whey. Biotechnol. Lett., 1986; 6(1):61-64

Table 1 Composition of a typical fermentation broth from cheese whey

Species	SYMBOL	CONCENTRATION (%m)
water	W	99.370
butanol	B	0.440
ethanol	E	0.012
acetone	A	0.100
butyric ac.	BA	0.045
acetic ac.	AA	0.006
lactose, protein, fat	-	trace

Table 2 Distribution factor of butanol between water and eicosanol and immiscibility limits of the eicosanol-water system

Butanol $Y_{X/W}$ [mole/mole]	SOLUBILITY [g _{water} /g _{eicosanol}]	SOLUBILITY [g _{eicosanol} /g _{water}]
65.5	0.07	/

Table 3 Equipment and manufacturing costs estimated under optimized conditions for a flow rate of fermentation broth of $5 \cdot 10^5$ ton/year

Equipment cost [\$]	$1.5 \cdot 10^6$
Distillation units	$7.7 \cdot 10^5$
Gas stripping tower	$2.7 \cdot 10^5$
Absorber tower	$2.2 \cdot 10^5$
Condenser	$2.0 \cdot 10^5$
Heat exchanger	$5.5 \cdot 10^4$
Manufacturing cost [\$/yr]	$1.1 \cdot 10^6$
Labour	$6.9 \cdot 10^5$
Wastewater treatment	$3.8 \cdot 10^5$
Water cooling	$4.5 \cdot 10^4$

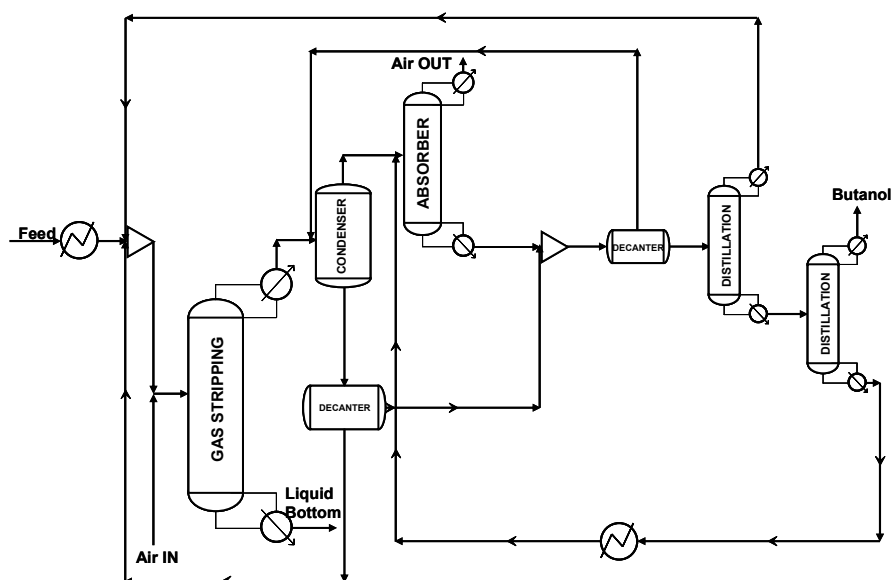


Fig. 1. Flowsheet for butanol recovery from ABE fermentation broth.

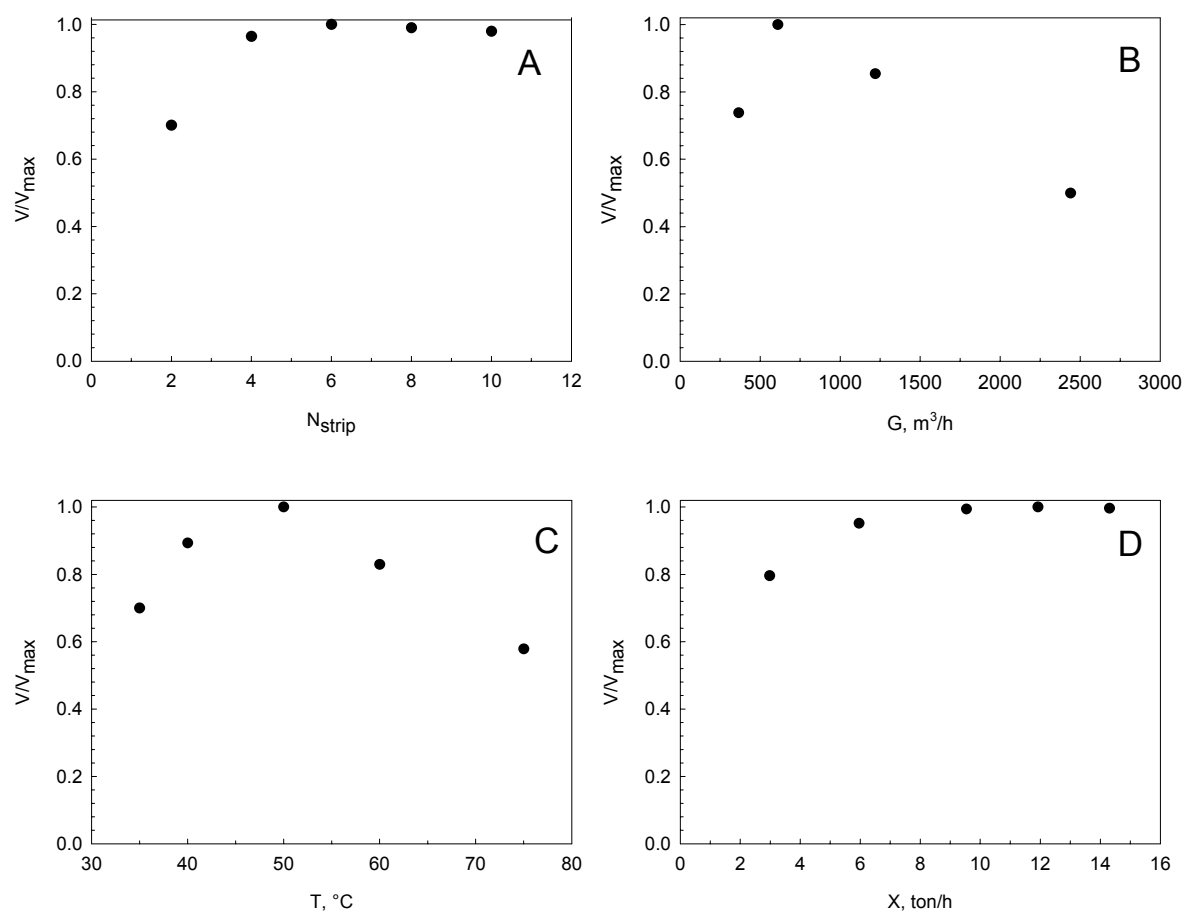


Fig. 2. Dimensionless venture profit as a function of selected design variables.

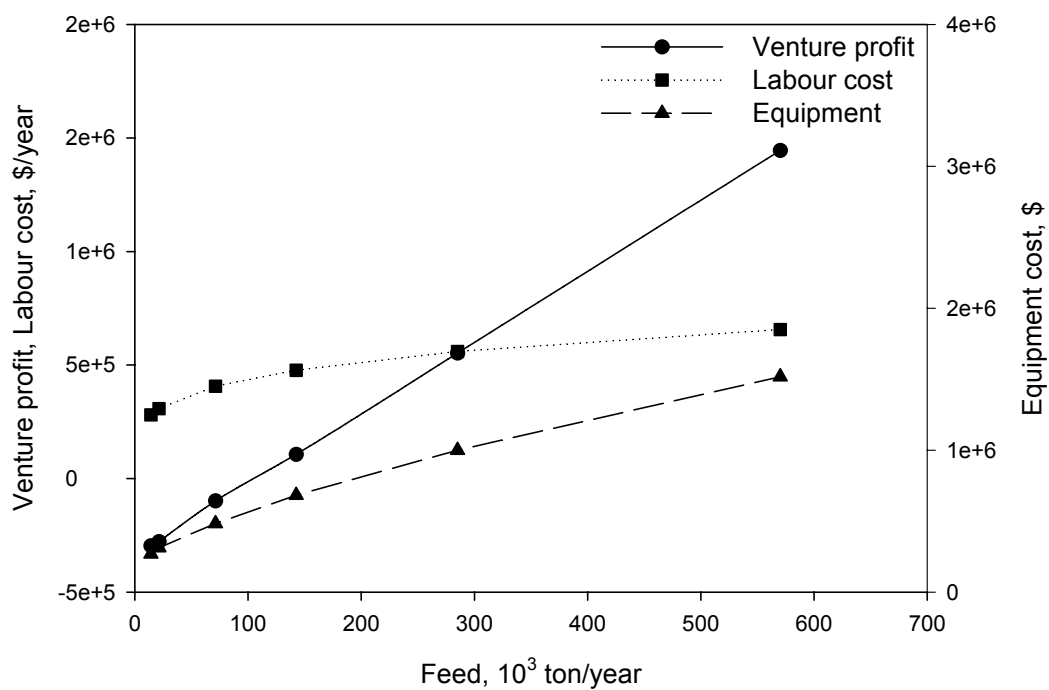


Fig. 3. Venture profit, capital investment and labour cost as a function of the processed flow rate.

7. CONCLUSIONS

The activities carried out within the PhD program have addressed the conversion of lactose to Acetone-Butanol-Ethanol by *Clostridium acetobutylicum* DSMZ 792. The study was articulated in three paths: i) the characterization of the ABE fermentation process as regards kinetics and yields; ii) the development of a continuous biofilm reactor for the ABE production; iii) the economic assessment of the process for the recovering and concentration of solvents.

Characterization of the ABE fermentation process as regards kinetics and yields.

The activity regarded both experimental and theoretical investigations.

A preliminary characterization of the specific growth rate of cells under acidogenesis conditions and the production rate of butanol at the onset of the solventogenesis phase was carried out under batch conditions. The investigation was successful and highlighted the relevant features of the transient conversion process: lactose conversion under acidogenesis conditions, pH threshold of the solventogenesis phase, raw estimation of the cell growth rate and butanol production rate.

Effective systems for the continuous fermentation under controlled conditions were successfully designed, set-up and operated. Two typologies of reactors were adopted: an isotherm CSTR equipped with a pH controller, and an isotherm CSTR equipped with a pH controller and a microfiltration unit. The first apparatus was adopted for the characterization under acidogenesis conditions, the second under solventogenesis conditions. The operating conditions were selected in order to characterize the fermentation process over a wide interval of pH (4.0-7.0) and substrate concentration (2-50 g/L). In particular acetic and butyric acids and ethanol, acetone and butanol were, if necessary, supplemented to the medium to characterize their effects on the fermentation process. The tests were characterized in terms of cells, metabolites and lactose concentrations.

The theoretical characterization regarded the assessment of the growth model of both the acidogenic cells and the solventogenic cells. A model for cell and metabolites yields was also assessed.

A multiproduct-inhibited growth model was proposed in agreement with Zeng *et al.* (1991 and 1994) and Tang *et al.* (1989). In particular, a new relationship was proposed to describe the effect of pH on the specific growth rate of *C. acetobutylicum*. The 16 model parameters were successfully assessed by adopting a successive approximation strategy. The model proposes as a tool to characterize the microorganism under a wide interval of operating conditions, even for acidogenic cells under solventogenesis phase.

A stoichiometric growth model was proposed. The model proposes as a tool to assess the mass fractional yield of both the biomass and the acids as a function of the operating conditions, expressed in terms of the specific growth rate. In particular, the maintenance model of Pirt (1982) was successfully adopted as a relationship between the specific growth rate and the energetic yield necessary for the biosynthesis.

The agreement between the theoretical assessments and the experimental data was successful over a wide interval of operating conditions. In particular, the model was able to simulate the fermentation process even under extreme operating conditions: i) huge presence of acids, properly supplemented; ii) huge presence of

solvents typically produced during the solventogenic phases. The latter conditions assume a relevant role in the investigation of the ABE production processes. In fact, under solventogenesis phase a fraction of cell produces biomass and acids.

Development of a continuous biofilm reactor for the ABE production.

The continuous conversion of lactose in Acetone-Butanol-Ethanol by *C. acetobutylicum* was studied. Several biofilm carriers were investigated and Tygon® rings were selected as optimal. A biofilm packed bed reactor was designed, set-up and successfully operated for more than one month. Operating conditions ranged were quite large: the pH ranged between 4.0 and 5.1, the dilution rate between 0.54 and 2.5 h⁻¹, the lactose concentration in the feed between 15 and 30 g/L. Reactor steady states were characterized in terms of solvents productivity and yields and of butanol selectivity.

The tests suggest that the reactor performances improve with D and pH within the intervals investigated. The maximum butanol productivity was the largest value reported in literature regarding the lactose conversion: 4.43 g/Lh. The butanol selectivity with respect solvents (88%_w) was among the largest value reported in literature. Moreover, the results highlight that the Tygon® is a potential carrier for butanol production.

Economic assessment of the process for the recovery and concentration of solvents.

A tentative design of a solvent recovery section for butanol production by fermentation from an arbitrary feedstock is presented. Proper selection of gas stripping, absorption and distillation units enables recovery of a high-purity (99.5% mol/mol) of butanol from relatively dilute (<0.5% mol/mol in butanol) fermentation broth. A preliminary assessment of the economics of the recovery process has been carried out with reference to a tentative economic scenario, suggesting that the process may be feasible provided that the throughput of the section exceeds a given threshold (around 250 ton/day in the simulated case). The low concentration of acetone and ethanol make their distillation recovery very expansive, emphasizing the necessity to enhance the process selectivity.

NOMENCLATURE

The nomenclature included in each manuscript is not reported.

AA, Ac, B,	acetic acid, acetone, butanol, butyric acid, ethanol, lactose,	g/L
BA, Et, L, X	and biomass concentration	
D	dilution rate	h ⁻¹
d ₁	biomass flow rate	g/h
f _n ⁱ	molar flow rate of production/uptake of product “i” with reference at reaction “n”	mole/Lh
w _n ⁱ	mass flow rate of production/uptake of product “i” with reference at reaction “n”	g/Lh
F _i	molar flow rate of production/uptake of product “i”	mole/Lh
W _i	mass flow rate of production/uptake of product “i”	g/Lh
Y _{ATP}	ATP yield	g _{DM} /mole _{ATP}
Greek Letters		
μ	specific growth rate	h ⁻¹

δ relative error on lactose consume

-

Subscripts

AA, Ac, B, BA, Et, L, X acetic acid, acetone, butanol, butyric acid, ethanol, lactose, biomass
A acidogenic
S solventogenic
D spore
T total

REFERENCES

- Afschar, A.S., Biebl H., Schaller K., Schugerl K., Production of acetone and butanol by *Clostridium acetobutylicum* in continuous culture with cell recycle. *Appl Microbiol Biotechnol* 1985, 22:394-398.
- Aivasidis, A., V.I. Diamantis, Biochemical reaction engineering and process development in anaerobic wastewater treatment. *Advances in Biochemical Engineering/Biotechnology*, 2005, 92:49-76
- Allison, D.G., Ruiz B., SanJose C., Jaspe C., Gilbert P., Extracellular products as mediators of the formation and detachment of *Pseudomonas fluorescens* biofilms. *FEMS Microbiol Lett* 1998, 167:179-184
- Andrews, G., Estimating cell and product yield, *Biotechnology and Bioengineering*, 1989, vol. 33, 256-265.
- Annachatre, A.P., Bhamidimarri S.M.R., Microbial attachment and growth in fixed-film reactors: process startup considerations. *Biotech Adv* 1992, 10:69-91.
- Applegate, D.H. and Bryers J.D., Effects of carbon and oxygen limitations and calcium concentrations on biofilm removal processes. *Biotech. Bioeng.* 1991, 37:17-25
- Bauchop, T. and S. R. Elsdon, The growth of microorganisms in relation to their energy supply, *J. Gen. Microbiol.*, 1960, vol. 23: 457-469
- Boyd, A. and Chakrabarty A.M., Role of alginate lyase in cell detachment of *Pseudomonas aeruginosa*. *Applied and environmental microbiology* 1994; vol. 60, 2355-9
- Bücks, J., Mozes N., Wandrey C., Rouxhet P.G. Cell adsorption control by culture conditions. *Appl Microbiol Biotechnol* 1988, vol. 29, 119-128.
- Carpentier, B., Cerf O., Biofilm and their consequences with particular reference to hygiene in the food industry. *J Appl Bacteriol* 1993, 75:499-511.
- Cao, N.J., Q. Xu, C.S. Chen, C.S. Gong, Cellulose Hydrolysis Using Zinc-Chloride as a Solvent and Catalyst, *Appl. Biochem. Biotechnol.*, 1994, vol. 45/46, 521-530.
- Cascone, R., Biobutanol - A Replacement for Bioethanol?, *Chem. Eng. Prog.*, 2008, August, S4-S9
- Characklis, W.G., McFeters G.A., Marshall K.C., Physiological ecology in biofilm systems. In *Biofilms Vol. 37*. Edited by: Characklis WG, Marshall KC. New York: John Wiley and Sons; 1990:67-72.
- Cooper, P.F., Atkinson B., (1981) *Biological Fluidized Bed Treatment of Water and Wastewater*. Publisher: Ellis Horwood Ltd., Chichester Engl.
- Costerton, JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM: Microbial biofilms. *Annu Rev Microbiol* 1995, vol. 49. 711-745.
- Costerton, J.W., Stewart P.S., Greenburg E.P., Bacterial biofilms: a common cause of persistent infections. *Science* 1999, vol. 284, 1318-1322.

- Cowan, M.M., Warren T.M., Fletcher M., Mixed species colonization of solid surfaces in laboratory biofilms. *Biofouling* 1991, vol. 3, 23-34.
- Crueger, W., Crueger C., Organic acids. In "Biotechnology" A textbook of industrial microbiology Sinauer Associates, Inc., Sunderland. MA; 1989.
- Davies, D.G., Chakrabarty AM, Geesey GG: Exopolysaccharide production in biofilms: substratum activation of alginate gene expression by *Pseudomonas aeruginosa*. *Appl Environ Microbiol* 1993, 59:1181-1186.
- Demirci, A., Pometto A.L. III, Ho K.L.G., Ethanol production by *Saccharomyces cerevisiae* in biofilm reactors. *J. Ind. Microbiol. Biotechnol.* 1997, vol. 19, 299-304.
- Desai, R.P., L.K. Nielsen, E.T. Papoutsakis, Stoichiometric modeling of *Clostridium acetobutylicum* fermentations with non-linear constraints, *Journal of Biotechnology*, 1999, vol. 71, 191-205.
- Donlan, R.M., Costerton JW: Biofilms: survival mechanisms of clinically relevant microorganisms, *Clin. Microbiol. Rev.*, 2002, vol. 15, 167-193.
- Donlan, R.M., Biofilms: microbial life on surfaces. *Emerg Inf Dis* 1992, 8(9):881-890.
- Dyr, J., J. Protiva, R. Praus, Formation of neutral solvents in continuous fermentation by means of *Clostridium acetobutylicum*, 1958, p 210-226. In I. Malek (ed.), Continuous cultivation of microorganisms. A symposium. Czechoslovakian Academy of Sciences, Prague.
- Ebener, J., T.C. Ezeji, N. Qureshi, H.P. Blaschek, 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. 2003
- Ennis, B.M., C.T. Marshall, I.S. Maddox, A.H.J. Paterson, Continuous product recovery by in-situ gas stripping/condensation during solvent production from whey permeate using *Clostridium acetobutylicum*, *Biotechnology Letters*, 1986, vol. 8, 725-730
- Ennis, B.M., I.S. Maddox, Use of *Clostridium acetobutylicum* P262 for production of solvents from whey permeate, *Biotechnology Letters*, 1985, vol. 7, 601-606
- Ezeji, T.C., N. Qureshi, H.P. Blaschek, Bioproduction of butanol from biomass: from genes to bioreactors, *Current Opinion in Biotech.*, 2007, vol.18, 220-227.
- Ezeji, T.C., N. Qureshi, H.P. Blaschek, Acetone-butanol-ethanol production from concentrated substrate: reduction in substrate inhibition by fed-batch technique and product inhibition by gas stripping, *Appl. Microbiol. Biotechnol.*, 2004, vol. 63, 653-658.
- Flickinger, M. C., S.W. Drew, *Bioprocess technology: fermentation, biocatalysis, and bioseparation*, Wiley-Blackwell, John Wiley & Sons Inc. 1999, vol 1 – 5.
- Forberg, C., Haggstrom L., Control of cell adhesion and activity during continuous production of acetone and butanol with adsorbed cells. *Enz Microbial Technol* 1985, vol. 7, 230-234.
- Gapes, J.R., The economics of the acetone-butanol fermentation: theoretical and market considerations. *J. Mol. Microbiol. Biotechnol.*, 2000, vol. 2, 27-32
- Gapes, J.R., The history of the acetone-butanol project in Austria. *J. Mol. Microbiol. Biotechnol.*, 2000, vol. 2, 5-8.
- Griffith, W.L., A.L. Compere, J. M. Googin, Novel neutral solvents fermentations, *Dev. Ind. Microbiol.*, 1983, vol. 24, 347-352
- Groot, W.J., G.H. Schoutens, P.N. Van Beelen, C.E. Van den Oever, N.W.F. Kossen. 1984. Increase of substrate conversion by pervaporation in the continuous butanol fermentation, *Biotechnol. Lett.*, 1984, vol. 6, 789-792.

- Hall, E.R., Biofilm reactors in anaerobic wastewater treatment. *Biotech. Adv.*, 1987, vol. 5, 257-269.
- Hartmanis, M.G.N., T. Klason, and S. Gatenbeck, Uptake and activation of acetate and butyrate in *Clostridium acetobutylicum*. *Appl. Microbiol. Biotechnol.*, 1984, vol. 20, 66-71
- Huang, W.C., Ramey D.E., Yang S.T., Continuous production of butanol by *Clostridium acetobutylicum* immobilized in a fibrous bed bioreactor. *Appl. Biochem. Biotechnol.*, 2004, vol. 113, 887-898.
- Jarzbowski, A.B., J.J. Malinowski, G. Goma and P. Soucaille, Analysis of continuous fermentation processes in aqueous two-phase systems, 1992, vol 7, 315-317
- Jones, D.T., D.R. Woods, Acetone-Butanol Fermentation Revisited, *Microb. Rev.*, 1986, Dec, 484-524
- Jui-shen Chiao, Zhi-hao Sun History of the Acetone-Butanol-Ethanol Fermentation Industry in China: Development of Continuous Production Technology *J. Mol. Microbiol. Biotechnol.*, 2007, vol. 13, 12-14
- Kolot, F.B., Immobilized cells for solvent production, *Process. Biochem.*, 1984, vol. 19, 7-13.
- Lenz, T.G., A.R. Moreira, Economic evaluation of the acetone butanol fermentation, *Ind. Eng. Prod. Res. Dev.*, 1980, vol. 19, 478-483.
- Lettinga, G, Roersma R, Grin P: Anaerobic treatment of raw domestic sewage at ambient temperature using a granular bed UASB reactor., *Biotechnol. Bioeng.*, 1983, vol. 25, 1701-1723.
- Lettinga, G., van Nelsen A.F.M., Hobma S.W., de Zeeuw W., Klapwijk A., Use of the upflow sludge blanket (USB) reactor concept for biological wastewater treatment, especially for anaerobic treatment, *Biotechnol. Bioeng.*, 1980, vol. 22, 699-734.
- Leung, J.C.Y., D.I.C. Wang, Production of acetone and butanol by *Clostridium acetobutylicum* in continuous culture using free cells and immobilized cells. *Proc. 2nd World Congr. Chem. Eng.*, 1981, vol. 1, 348-352.
- Loung, J.H.T., Kinetics of ethanol inhibition in alcohol fermentations, *Biotechnol. Bioeng.*, 1985, vol. 27, 280-285
- Linden, J.C., A.R. Moreira, T.G. Lenz, 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. and L.T. Fan, Downstream Process Synthesis for Biochemical Production of Butanol, Ethanol, and Acetone from Grains: Generation of Optimal and Near-Optimal Flowsheets with Conventional Operating Units *Biotechnol. Prog.* 2004, vol. 20, 1518-1527
- Maddox, I.S., Qureshi N., McQueen J., Continuous production of 2,3-butanediol from whey permeate using cells of *Klebsiella pneumoniae* immobilized on to bonechar, *New Zealand J. Dairy Sci. Technol.*, 1988, vol. 23, 127-132
- Maddox, I.S., The acetone butanol ethanol fermentation: recent progress in technology, *Biotechnol. Genetic Eng. Reviews* 1989, vol. 7, 190-220.
- Marchal, R., M. Rebeller, and J. P. Vandecasteele, Direct bioconversion of alkali-pretreated straw using simultaneous enzymatic hydrolysis and acetone-butanol fermentation. *Biotechnol. Lett.*, 1984, vol. 6, 523-528
- Mehaia, M.A., Cheryan M., Ethanol production in a hollow fiber bioreactor using *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.*, 1984, vol. 20, 100-104

- Meyer, C.L., E.T. Papoutsakis, Continuous and biomass recycle fermentations of *Clostridium acetobutylicum*. Part 1: ATP supply and demand determines product selectivity, *Bioprocess Engineering*, 1989, vol. 4, 1-10.
- Meyer, A., Wallis F.M., Development of microbial biofilms on various surfaces for the treatment of heavy metal containing effluents, *Biotechnol. Tech.*, 1997, vol. 11, 859-863.
- Mutschlechner, O., H. Swoboda, J.R. Gapes, Continuous Two-Stage ABE-Fermentation using *Clostridium beijerinckii* NRRL B592 Operating with a Growth Rate in the First Stage Vessel Close to its Maximal Value, *J. Mol. Microbiol. Biotechnol*, 2000, vol. 2, 101-105.
- Mc Donogh, R., Schaule G., Flemming H.C., The permeability of biofouling layers on membranes, *J. Membr. Sci.*, 1994, vol. 87, 199-217
- Nakas, J.P., M. Schaedle, C.M. Parkinson, C.E. Coonley, S.W. Tanenbaum., System development for linkedfermentation production of solvents from algal biomass, *Appl. Environ. Microbiol.*, 1983, vol. 46, 1017-1023
- Nicolella, C., van Loosdrecht M.C.M., Heijnen S.J., Particle-based biofilm reactor technology, *Trends in Biotechnol. (TIBTECH)*, 2000, vol.18, 312-320.
- Nimcevic, D., and J.R. Gapes, The acetone-butanol fermentation in pilot plant and pre-industrial scale, *J. Mol. Microbiol. Biotechnol.*, 2000, vol. 2, 15-20
- Olivieri, G., A. Marzocchella, A. Di Donato, P. Salatino, Bioreactors for azo-dye conversion, in "The Handbook of Environmental Chemistry: Biodegradation of Azo Dyes", Ed. H.A. Erkurt, Springer 2009, in press
- Oudshoorn, A., Luuk A. M. van der Wielen, and Adrie J. J. Straathof, Assessment of Options for Selective 1-Butanol Recovery from Aqueous Solution, *IECR*.....
- Pierrot, P., Fick M., Engasser J.M., Continuous acetone butanol fermentation with high productivity by cell ultrafiltration and recycling, *Biotechnol. Lett.*, 1986, vol. 8, 253-256.
- Papoutsakis, E.T., Engineering solventogenic clostridia ,*Current Opinion in Biotechnology*, 2008, vol. 19, 420–429
- Papoutsakis, E.T., Equations and calculations for fermentations of butyric acid bacteria, *Biotechnology and Bioengineering*, 1984, vol. 26, 174-187
- Pirt, S.J., Maintenance Energy: a General Model for Energy-Limited and Energy-Sufficient Growth, *Arch. Microbiol.*, 1982, vol. 133, 300- 302
- Pierrot, P., N. Fick, J.M. Engasser, Continuous Acetone-Butanol Fermentation With High Productivity By Cell Ultrafiltration and recycling, *Biotechnology Letters*, 1986, vol. 8, 253-256
- Qureshi N, B.A. Annous, T.C. Ezeji, P. Karcher and I.S. Maddox, Biofilm reactors for industrial bioconversion processes: employing potential of enhanced reaction rates, *Microbial Cell Factories*, 2005, vol. 4, 24
- Qureshi, N., Karcher P., Cotta M., Blaschek H.P., High-productivity continuous biofilm reactor for butanol production, *Appl. Biochem. Biotechnol.*, 2004, vol. 113–116, 713-721.
- Qureshi, N., Blaschek H.P., Evaluation of recent advances in butanol fermentation, upstream, and downstream processing, *Bioproc. Biosys. Eng.*, 2001, vol. 24, 219-226.
- Qureshi, N., J. Schripsema, J. Lienhardt, H.P. Blaschek, Continuous solvent production by *Clostridium beijerinckii* BA101 immobilized by adsorption onto brick, *World J. Microbiol. Biotechnol.*, 2000, vol. 16, 377-382
- Qureshi, N., Maddox I.S., Integration of continuous production and recovery of solvents from whey permeate: use of immobilized cells of *Clostridium*

- acetobutylicum in fluidized bed bioreactor coupled with gas stripping. *Bioprocess Engineering* 1991, vol. 6, 63-69.
- Qureshi, N., I.S. Maddox, Reactor design for the ABE fermentation using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar, *Bioprocess. Eng.* 1988, vol. 3, 69-72
- Qureshi, N., Maddox I.S., Continuous solvent production from whey permeate using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar, *Enz. Microbial. Technol.*, 1987, vol. 9, 668-671
- Qureshi, N., Maddox IS: Novel bioreactors for the ABE fermentation using cells of *Clostridium acetobutylicum* immobilized by adsorption onto bonechar. In *Fermentation technologies: Industrial applications* Edited by: Yu PL. London: Elsevier Appl Sci Publ; 1990.
- Qureshi, N., Paterson AHJ, Maddox IS: 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.*, 1988, vol. 29, 323-328
- Rajni, H.K., Aqueous two-phase systems, *Molecular Biotechnology*, 2001, vol. 19 269-277
- Rogers, P., Genetics and biochemistry of *Clostridium* relevant to development of fermentation processes, *Appl. Microbiol.* 1984, vol. 31, 1-60.
- Ross, D., The acetone-butanol fermentation. *Prog. Ind. Microbiol.*, 1961, vol. 3, 73-85.
- Russo, M.E., "Intensification of Bioconversion Processes: Design and Development of Reactors with High Biocatalyst Loading", PhD thesis, Università degli Studi di Napoli *Federico II*, 2008
- Sarrafzadeh, M.H., L. Belloy, G. Esteban³, J.M. Navarro, C. Ghommidh, Dielectric monitoring of growth and sporulation of *Bacillus thuringiensis*, *Biotechnology Letters*, 2005, vol. 27, 511-517,
- Schuster, K.C., R. Goodacre, J.R. Gapes, M.Young, Degeneration of solventogenic *Clostridium* strains monitored by Fourier transform infrared spectroscopy of bacterial cells, *Journal of Industrial Microbiology & Biotechnology*, 2001, vol. 27, 314-321.
- Seghezzo, L., Zeeman G., van Lier JB, Hamelers H.V.M., Lettinga G., A review: The anaerobic treatment of sewage in UASB and EGSB reactors. *Bioresource Technol* 1998, vol. 65, 175-190.
- Shinto, H., Y. Tashiro, M. Yamashita, G. Kobayashi, T. Sekiguchi, Taizo Hanai, Yuki Kuriya, Masahiro Okamoto, Kenji Sonomoto, Kinetic modeling and sensitivity analysis of acetone-butanol-ethanol production, *Journal of Biotechnology*, 2007, vol. 131, 45-56.
- Sponza, D.T., Anaerobic granule formation and tetrachloroethylene (TCE) removal in an upflow anaerobic sludge blanket (UASB) reactor, *Enz. Microbial. Technol.*, 2001, vol. 29, 417-427.
- Stoodley, P., Sauer K., Davies D.G., Costerton J.W., Biofilms as complex differentiated communities, *Annu. Rev. Microbiol.*, 2002, vol. 56, 187-209.
- Taras, M., Hakansson K., Guieysse B., Continuous acetonitrile degradation in packed-bed bioreactor, *Appl. Microbiol. Biotechnol.*, 2005, vol. 66, 567-574.
- Tashiro, Y., K. Takeda, G. Kobayashi, K. Sonomoto, A. Ishizaki, S. Yoshino, High butanol production by *Clostridium saccharoperbutylacetonicum* N1-4 in fed-batch culture with pH-Stat continuous butyric acid and glucose feeding method, *Journal of Bioscience and Bioengineering*, 2004, vol. 98, 263-268

- Tay, A., Yang S.T., Production of L-(+)-lactic acid from glucose and starch by immobilized cells of *Rhizopus oryzae* in a rotating fibrous bed bioreactor, *Biotechnol. Bioeng.*, 2002, vol. 80, 1-12
- The Brew Project (2006) "Medium and Long-term Opportunities and Risks of the Biotechnological Production of Bulk Chemicals from Renewable Resources – The Potential of the White Biotechnology". Final report European Commission's GROWTH Programme (DG Research) Utrecht University.
- towers: mechanisms and design, C.T.I. J., 1994, vol. 15, 10-25.
- Tsuno, H., Kawamura M, Somiya I: Anaerobic degradation of pentachlorophenol (PCP) in biological expanded-bed reactor, *Water Sci. Technol.*, 1996, vol. 34, 335-344
- Tyagi, R.D., Ghose T.K., Studies on immobilized *Saccharomyces cerevisiae*. I. Analysis of continuous rapid ethanol fermentation in immobilized cell reactor, *Biotechnol. Bioeng.*, 1982, vol. 24, 781-795
- Varga, E., Klinke, H.B., Reczey K., Thomsen A.B., High solid simultaneous saccharification and fermentation of wet oxidized corn stover to ethanol, *Biotechnol. Bioeng.*, 2004, vol. 88, 567–574
- Veeresh, G.S., Kumar P., Mehrotra I., Treatment of phenol and cresols in upflow anaerobic sludge blanket (UASB) process, A review *Water Research*, 2005, vol. 39,154-170.
- Villadsen, J., Innovative technology to meet the demands of the white biotechnology revolution of chemical production, *Chem. Engng. Sci.*, 2007, vol. 62, 6957-6968
- Voget, C.E., C.F. Mignone, R. J. Ertola, Butanol production from apple pomace. *Biotechnol. Lett.*, 1985, vol. 7, 43-46.
- Voget, C. E., C. F. Mignone, R. J. Ertola, Influence of temperature on solvents production from whey. *Biotechnol. Lett.*, 1989, vol. 7, 607-610.
- Wang, D. I. C., C. L. Cooney, A. L. Demain, and P. Dunhill, *Fermentation and Enzyme Technology*, 1979, John Wiley & Sons, Inc., New York.
- Watnick, P., Kolter R., Biofilm, City of microbes, *J. Bacteriol.*, 2000, vol. 182, 2675-2679.
- Welsh, F.W., Williams R.E., Veliky I.A., Solid carriers for a *Clostridium acetobutylicum* that produced acetone and butanol, *Enz. Microbial. Technol.*, 1987, vol. 9, 500-502.
- Welsh, F.W., I.A. Veliky, The metabolism of lactose by *Clostridium acetobutylicum*, 1986, vol. 8, 43-46
- Welsh, F. W., I. A. Veliky, Production of acetone butanol from acid whey, *Biotechnol. Lett.*, 1984, vol. 6, 61-64.
- Wiley, A. S., M. J. Johnson, E. Mc Coy, W. H. Peterson, The fermentation of waste sulfite liquor. *Ind. Eng. Chem.*, 1941, vol. 35, 606-610.
- Wobus, A., Ulrich S., Roske I., Degradation of chlorophenols by biofilms on semi-permeable membranes in two types of fixed bed reactors, *Water Sci. Technol.*, 1995, vol. 32, 205-212.
- Woods, H. G., R. W. Brown, and C. H. Werkman, Mechanism of the butyl alcohol fermentation with heavy carbon acetic and butyric acids and acetone, *Arch. Biochem.*, 1945, vol. 6, 243-261.
- Woolard, C.R., Irvine R.L., Biological treatment of hypersaline wastewater by a biofilm of halophilic bacteria, *Water Environ. Res.*, 1994, vol. 66, 230-235
- Zeng, A.-P. Pathway and kinetic analysis of 1,3-propanediol production from glycerol fermentation by *Clostridium butyricum*, *Bioproc. Eng.*, 1996, vol. 14, 169-175