This is an electronic reprint of the original article.
This reprint may differ from the original in pagination and typographic detail.

Bankar, Sandip B.; Survase, Shrikant A.; Singhal, Rekha S.; Granström, Tom

Continuous two stage acetone-butanol-ethanol fermentation with integrated solvent removal using *Clostridium acetobutylicum* B 5313

Published in:
Bioresource Technology

DOI:
[10.1016/j.biortech.2011.12.005](https://doi.org/10.1016/j.biortech.2011.12.005)

Published: 01/01/2012

Document Version
Peer reviewed version

Published under the following license:
CC BY-NC-ND

Please cite the original version:
Bankar, S. B., Survase, S. A., Singhal, R. S., & Granström, T. (2012). Continuous two stage acetone-butanol-ethanol fermentation with integrated solvent removal using *Clostridium acetobutylicum* B 5313. *Bioresource Technology*, 106, 110-116. <https://doi.org/10.1016/j.biortech.2011.12.005>

Continuous two stage acetone-butanol-ethanol fermentation with integrated solvent removal using *Clostridium acetobutylicum* B 5313

Sandip B. Bankar^{1,2}, Shrikant A. Survase^{*1}, Rekha S. Singhal², Tom Granström¹

¹Aalto University, School of Science and Technology, Department of Biotechnology and Chemical Technology, POB 16100, 00076 Aalto, Finland

²Department of Food Engineering and Technology, Institute of Chemical Technology, Matunga (E), Mumbai 400 019, India.

* Corresponding author:

Dr. Shrikant A. Survase;

Phone: +358-400368375;

Fax: +358- 9 462 373;

Email: shrikant.survase@aalto.fi

Abstract

The objective of this study was to optimize continuous acetone-butanol-ethanol (ABE) fermentation using two stage chemostat system integrated with liquid-liquid extraction of solvents produced in first stage. This minimized end product inhibition by butanol and subsequently enhanced glucose utilization and solvent production in continuous culture of *Clostridium acetobutylicum* B 5313. During continuous two-stage ABE fermentation, sugarcane baggase was used as cell holding material for the both stages and liquid-liquid extraction was performed using oleyl alcohol and decanol mixture. An overall solvent production of 25.32 g/L (acetone 5.93 g/L, butanol 16.90 g/L and ethanol 2.48 g/L) was observed as compared to 15.98 g/L in single stage chemostat with highest solvent productivity and solvent yield of 2.5 g/L.h and of 0.35 g/g, respectively. Maximum glucose utilization (83.21 %) at dilution rate of 0.05 1/h was observed as compared to 54.38 % in single stage chemostat.

Key words: Butanol; two stage chemostat; oleyl alcohol; sugar cane baggase; *Clostridium acetobutylicum*

1. Introduction

Continuous depletion of petroleum fuel-reserves and various environmental issues like greenhouse effects, global warming, and climate change are the current issues to be resolved worldwide. This decline of reserve, the rising price and the concerns on the environmental impact of petroleum-based fuel have initiated interest in renewable biofuels (Ramos et al., 2009; Koh and Ghazoul, 2008). The acetone-butanol-ethanol (ABE) fermentation process continues to receive attention as a source of fuel and chemical feedstock based on renewable resources. The traditional batch fermentation process, however, suffers from various problems which impede its commercial development. End-product inhibition, low product concentration, large volumes of fermentation broth, the requirements of large bioreactors, and high cost involved in generating the steam required to distill the fermentation products from broth has largely contributed to the decline in fermentative ABE production. Low reactor productivity and the severe product inhibition which accompanies the process, limits the ABE concentration in the broth up to 20 g/L. In Clostridial ABE fermentation, butanol inhibition limits product titer which in turn contributes to the high cost of product recovery (Ezeji et al., 2005). These problems can be solved either by genetic manipulation of the Clostridium bacterium or by advanced bioprocess developments of ABE production and recovery. Process development for ABE fermentation with marked improvements in reactor productivity have been reported *viz.* continuous culture and immobilized cells culture techniques (Qureshi and Maddox, 1995). However, product recovery from dilute solutions and disposal of large volumes of effluent are the drawbacks with these systems. Recycling the reactor effluent so as to achieve complete sugar utilization with continues removal of the inhibitory solvents may lead to higher product concentrations, as has been attempted in the present study. Therefore, *in situ* solvent extractive fermentation has been proposed as one of the approaches to minimize butanol inhibition and increase product titer in fermentation broth (Ezeji et al., 2005; Ishizaki et al., 1999; Grobбен et al., 1993).

In order to improve the extraction process, number of solvents have been identified and tested on the basis of their selectivity for butanol and biocompatibility with the fermentative organism (Shukla et al., 1988; Job et al., 1989). Among these solvents, oleyl alcohol (Qureshi and Maddox, 1995; Davison and Thompson, 1993), decanol (Evans and Wang, 1988), dibutyl phthalate (Qureshi and Maddox, 1995) and polypropylene glycol (Barton and Daugulis, 1992) have been extensively used at the laboratory scale for *in situ* solvent extraction. Methylated crude palm oil (Ishizaki et al., 1999) and methylated sunflower oil (Grobбен et al., 1993) as a butanol extractant have also been successfully studied to maintain the low butanol level in fermentation broth.

The productivity of continuous cultivation can be increased to values between 0.5 and 1.0 g/L.h in a culture of freely suspended cells (Maddox, 1989). Modest increase in productivity can be achieved by increasing the cell concentration in

medium. According to several methods described in the literature, cell immobilization is found to be most advantageous method for continuous ABE fermentation. The use of carrier material and formation of cell aggregates are useful to increase the biomass concentration and therefore the productivity of microbial process (Beefink et al., 1988). Our previous study of continuous production of ABE with SO₂–ethanol–water spent liquor from spruce chips has successfully employed immobilized culture in the column to enhance the solvent productivity (Survase et al., 2011a).

Attempts to improve the productivity of ABE fermentation using two-stage continuous bioreactor (Mutschlechner et al., 2000; Bahl et al., 1982) and *in situ* recovery in continuous bioreactor (Qureshi and Maddox; Gapes et al., 1996) have been reported. These approaches have given low productivities of the order of 0.25 g/L.h to 1.5 g/L.h (Gapes et al., 1996; Qureshi and Maddox). We hypothesized that an inclusion of *in situ* extraction of ABE between the two stages of continuous extraction would overcome the toxicity of the solvent system to the organism and also increase the productivity by better utilization of the substrate. Besides, it would enable the reactor to be used continuously for a longer time. This approach has not been used so far for ABE fermentation. The present work planned in this direction and aimed to achieve and maintain the conditions which allow for degeneration-free, long term stable continuous two stage cultivation of *Clostridium acetobutylicum* B 5313 with a high overall solvent productivity. Sugarcane bagasse as a carrier material was used in both the stages. A liquid-liquid extraction module was integrated in between the two stages to counter the product inhibition, and allow higher productivities with maximum sugar utilization.

2. Materials and methods

2.1 Organism, maintenance and inoculum preparation

C. acetobutylicum B 5313 (DSM 792, ATCC 824) was obtained from Russian National Collection of Industrial Microorganisms at the Institute of Genetics and Selection of Industrial Microorganisms (Moscow, Russia). Initially, sporulated cells were activated by heat shock at 80°C for 10 min. The activated spore culture (2.5 ml) was inoculated in 100 ml sterile reinforced clostridia medium (RCM) in 125 ml air tight, anaerobic glass bottles and grown for 20 h at 37°C. RCM medium contained (g/L) meat extract, 10; peptone, 5.0; yeast extract, 3.0, glucose, 30; starch, 1.0; sodium chloride, 5.0; sodium acetate, 3.0; L-cysteine, 0.50 and pH was adjusted to 6.5.

2.2. Production medium

The production medium reported by Monot et al. (1982) was used in present study, which contained (g/L): glucose, 60; ammonium acetate, 2.2; K₂HPO₄, 0.5; KH₂PO₄, 0.5; *p*-aminobenzoic acid, 0.1; thiamine hydrochloride, 0.1; biotin, 0.01; FeSO₄, 0.01; MnSO₄·H₂O, 0.01; MgSO₄, 0.2; NaCl, 0.01. The medium pH was adjusted to 6.5 with HCl. The medium was then autoclaved at 10⁵ Pa (121°C) for 20 min and cooled.

2.3. Continuous fermentation with immobilized cells

In batch fermentation, reactor productivity is in the order of 0.35– 0.40 g/L.h (Qureshi and Maddox, 1995). Such low reactor productivity requires large reactor volumes which results in high capital and operational costs. In order to reduce the cost of production, high reactor productivity is desirable, which can be achieved using immobilized cell reactors or membrane cell recycle reactors. Since immobilized cell reactors are cheaper and are simple to operate, this technique was employed to achieve high reactor productivity. Sugarcane baggase was used as a support material for cells during immobilized cell fermentation.

2.3.1. Single-stage chemostat cultivation

Single-stage chemostat cultivations were carried out in 1 L jacketed glass bioreactor with fermentation volume of 500 ml. The bioreactor contained sugarcane bagasse (size, 5 to 10 mm), packed in nylon mesh basket which served as a carrier material for bacterial cells. The carrier material to liquid ratio was 1:4 (previously optimized, data not shown). The bioreactor was inoculated with 10 % inoculum of highly motile cells of *C. acetobutylicum* B 5313. The fermentation was allowed to proceed in batch mode for 24 h, after which fermentation feed medium was continuously pumped into the bioreactor at different dilution rates, wherever mentioned. The cells were adsorbed onto the sugarcane bagasse during batch growth, before starting continuous fermentation. The working volume of the fermenter was kept constant by removing excess medium from the bioreactor at a rate equal to the feed rate. Fresh medium was continuously introduced into the bioreactor using a peristaltic pump (Watson-Marlow Ltd., Falmouth, England). Dilution rates were varied from 0.05 L/h and 0.70 L/h with reactor temperature of 37 °C. During the course of fermentation, samples were collected at regular intervals and analyzed for biomass, acetone, butanol, ethanol, residual sugar and acids. Unless otherwise stated, all continuous fermentations were carried out in duplicates and results reported are average of two fermentations. Samples for biomass and product analysis were taken after five reactor volume for two consecutive days at steady state condition. The steady state was confirmed with stable biomass and product values at a specific dilution rate.

2.3.2. Two-stage chemostat integrated with liquid-liquid extraction

Two stage chemostat set up was adopted from our previous study (Survase et al., 2011b) with some modification. The experimental setup with two stage bioreactor (different volume in both) were carried out with sugarcane bagasse as cell holding material and integrated with liquid-liquid extraction module for *in situ* solvent removal. The bioreactors (stage 1 and stage 2) contained sugarcane bagasse (size 5 to 10 mm), packed in nylon mesh basket which served as a carrier material (ratio with liquid phase was 1:4) for bacterial cells. The assembly consisted of four glass jacketed bioreactors with total volume 1 L. Bioreactors were arranged sequentially and designated as stage one (R1), extractor (E), settling vessel (S) and stage two (R2) (Fig. 1). Culture volumes (including medium and carrier material) in two stages R1 and R2 were maintained as 350 mL and 700 mL, respectively. Fresh medium was continuously introduced into R1

with the help of peristaltic pump. Dilution rate of R1 was varied in between 0.05 and 1.0 1/h which resulted in dilution rate of 0.025 to 0.5 1/h in R2.

The bioreactors were inoculated with 10 % v/v highly motile cells of *C. acetobutylicum* B 5313. Fermentation was allowed to proceed in the batch mode for 24 h, after which fermentation feed medium was continuously pumped into R1 at different dilution rates as mentioned. Steady state was confirmed with stable biomass and product values at specific dilution rate. Samples for biomass and product analysis were taken after five working volume change at steady state conditions and for two consecutive days. All the vessels were maintained at 37 °C and medium from both the bioreactors were agitated at 200 rpm.

An extraction vessel (E) contained the extractant (oleyl alcohol:decanol, 4:1) to remove the solvents generated during continuous cultivation from R1. Culture broth and extractant were mixed by continuous agitation (400 rpm) with the help of magnetic stirrer and volume of extractor was maintained at 500 ml by maintaining the flow rate to separator and back flow of extractant from separator to extractor (Fig. 1). Mixture was allowed to separate into organic phase and aqueous phase in separator (S) and latter was pumped to the R2 where further fermentation was carried out using immobilized cells. The organic phase in separator was recycled to the extraction vessel and overall volume in R2 (medium and carrier material) was maintained upto 700 mL. Working volume of all the bioreactors were kept constant by removing extra medium with peristaltic pumps. All the physiological and nutritional parameters were kept same for both the bioreactors.

2.4. Optimization of extraction parameters

The distribution coefficient, extraction efficiency and selectivity of butanol was determined with synthetic broth and used for further studies. Literature reports on using oleyl alcohol and decanol in combination; for the extraction of butanol is available. Butanol is more soluble in the oleyl alcohol-decanol mixture than in the aqueous (fermentation broth) phase; therefore, butanol selectively concentrates in the organic phase. Different parameters like oleyl alcohol to decanol ratio, broth to extractant ratio and extraction time were also optimized in batch studies and were used for continuous experiments as an integrated system.

2.5. Analytical methods

2.5.1. Determination of substrates and products

Solvents (*n*-butanol, acetone and ethanol) and acids (acetic acid and butyric acid) were quantified on gas chromatography (Survase et al., 2011b). The gas chromatograph (Helwett Packard series 6890) equipped with a flame ionization detector and DB-WAXetr capillary column (30m × 0.32 mm × 1 µm) was used in this study. Injector temperature was 200 °C and detector temperature was maintained at 250 °C, with injection volume of 10 µl.

Glucose was determined by high-performance liquid chromatography (Bio-Rad Laboratories, Richmond, Calif.), equipped with an Inores S 259-H column (Inovex, Vienna, Austria) packed with Inores cation exchanger (particle size, 9 mm). The column was heated at 70°C, and the eluent (0.01 M H₂SO₄) was circulated with a flow rate of 0.60 mL/min. A cellobiose (Roth, Karlsruhe, Germany) solution was added

to the samples as an internal standard. A refractive index detector (model 1755; Bio-Rad) was used for quantification.

2.5.2. Calculation of bioprocess parameters

Dilution rate (1/h) was calculated by dividing flow rate with the working volume of bioreactor. Overall dilution rate was calculated by dividing flow rate of feed into the bioreactor with total volume of both the bioreactors. Overall solvent productivity in g/L.h during continuous cultivation of solvent producing *Clostridia* was expressed as g/L of total solvents multiplied by dilution rate (1/h). Substrate utilization (%) was determined with initial glucose concentration of the feed which was varied for R1 and for R2. Solvent yield was calculated by dividing total solvents in g/L by utilized substrate in g/L. The overall productivity and solvent yields were calculated by considering the total solvent production and glucose utilized in R1 and R2.

2.8. Effect of solvent extraction on glucose consumption

Higher glucose utilization in batch culture is limited by the end product inhibitory effect of butanol. Effect of integrated butanol removal on glucose consumption by *C. acetobutylicum* B 5313 in ABE fermentation was studied. The results for single stage continuous fermentation and two-stage continuous fermentation were compared by considering the fact that, input glucose was different for both the reactors in two stage fermentation. In two-stage continuous fermentation, the glucose feed concentration was high in R1 and low in R2, and hence the percent glucose consumption was compared with the dilution rates and observed for maximum utilization by microorganisms.

3. Results and discussion

Previous studies in our laboratories with *C. acetobutylicum* showed the solvent production to begin at the end of exponential phase (after six hours) and reach a stationary phase after 48 h fermentation in batch study (unpublished data). Low solvent productivity and yield with severe product inhibition propelled the necessity for continuous ABE fermentation with integrated extraction assembly. Continuous cultures with immobilized biomass or biomass retention are reported to give higher solvent productivities due to higher dilution rates. Solvent productivity has a big impact on capital cost of the operation. A two-fold increase in productivity reduces capital expenditure by approximately 20% together with significant reductions in operating costs (Green, 2011). Zhang et al. (2009) observed the highest solvent productivity and solvent yield as 5 g/L.h and 0.32 g/g with maximum dilution rate 1.0 1/h when cells were immobilized on corn stalk. The two-stage concept has also been successfully applied to other complex fermentation process such as methane production (Qureshi et al., 2000; Zhang et al., 2009).

3.1. Optimization of extraction parameters

Butanol extraction with liquid–liquid extraction has been reported to extract butanol from the fermentation broth without removing substrates, water or nutrients (Zhang, et al., 2009). Oleyl alcohol has been the extractant of choice among various researchers as it showed good extraction efficiency with non-toxicity towards

the cells (Ezeji et al., 2006; Karcher et al., 2005). Decanol has also been preferred as an extractant by various researchers due to its higher butanol distribution coefficient over oleyl alcohol; however, it was found to be toxic to the microorganisms when used alone (Assobhei et al., 1998; Qureshi et al., 2005). In this context, the optimization of these two consequences (toxicity and distribution coefficient) resulted in the use of a mixed extractant containing 20% decanol in oleyl alcohol which was found to be suitable for maximum butanol extraction with less toxicity, and the results are in agreement with other researchers (Qurashi et al., 2005; Kumar and Gayen, 2011). The optimum ratio of broth to extractant was found to be 1:1.5 and results remained unchanged at higher proportion of the extractant (data not shown). The contact time for efficient extraction was found to be optimum at 30 min, and remained unchanged thereafter from 60 min up to 8 h (data not shown).

3.2. Single stage continuous production of ABE

Clostridial ABE fermentation possesses sequential acidogenic and solventogenic biphasic fermentation. In the acidogenic phase which usually takes place in the exponential growth phase, substrate is converted into acetic acid, butyric acid, H₂ and CO₂. During the solventogenic phase which usually occurs in the stationary phase, acetic acid, and butyric acid are reassimilated and converted into acetone, butanol, and ethanol (Zverlov et al., 2006, Matta-el-Ammouri et al., 1987).

Effect of different dilution rate on ABE production is shown in Fig 2. The solvent productivity increased with an increase in the dilution rate up to a dilution rate of 0.6 1/h and remained constant till 0.7 1/h, although the solvent yields decreased continuously. Total solvent production of 3.54 g/L (acetone 0.86 g/L, ethanol 0.87 g/L and butanol 1.82 g/L) was observed at dilution rate 0.60 1/h. The maximum solvent productivity (2.12 g/L.h) resulted in solvent yield of 0.25 g/g glucose which was observed at a dilution rate 0.6 1/h. Lee et al. (2008) immobilized *C. beijerinckii* on porous polyvinyl alcohol media at a dilution rate 0.4 1/h with overall butanol productivity and yield was 0.40 g/L.h. and 0.44 g/g glucose, respectively. For maximum solvent productivity, the culture could utilize only 24.14 % of initial glucose and very large fraction remained unconsumed at higher dilution rate. Bahl et al. (1982) reported *C. acetobutylicum* to ferment 300 mmol of glucose to 130 mmol of butanol and approximately 20% glucose to remain in the medium. In a single-stage continuous culture, when wood pulp was added as a cell holding material, the solvent productivity was shown to increase from 0.47 to 5.52 g/L.h with the yield of 54% from glucose. An overall solvent concentration of 7.51 g/L with maximum solvent productivity as 0.84 g/L.h was obtained with two-stage continuous culture (Survase et al., 2011b). Survase et al. (2011a) further employed continuous immobilized columns for ABE production owing to its high pressure holding capacity and higher dilution rate sustainability. Total solvent concentration of 12 g/L and maximum solvent productivity (4.86 g/l h) with yield of 0.27 g/g was obtained in column reactor. Since the cells were immobilized on sugarcane baggase, higher dilution rate was not a hindrance for higher production of solvents, in the present study. Besides, the cells also remained intact unlike suspended cells.

3.3. Two-stage ABE fermentation with integrated liquid-liquid extraction

With the objective of increasing the productivity, yield and maximum utilization of glucose, the production of solvents using two-stage chemostat has been proposed and evaluated as a method of choice by various researchers during cultivation of free suspended cells in stirred tank bioreactors (Gapes et al., 1996; Godin and Engasser, 1989, Mutschlechner et al. 2000). Reactors R1 and R2 were operated with integrated product recovery mode using oleyl alcohol and decanol (4:1) as the extractant at different dilution rates as used in single stage bioreactor (Fig. 1). The overall dilution rates and product formed in both the bioreactors are shown in Fig 3, while the consumption of glucose is shown in Fig. 4.

Input glucose concentration was low in R2 as compared to R1 and hence the fermentation volume in R2 was kept double as compared to R1, so that it would get higher residence time in R2. Volume for extractor and separator vessel was maintained at 500 mL and excess liquid was pumped into another reactor as shown in Fig. 1. Solvent production and glucose consumption profile in R1 was very similar to that with the single stage continuous experiments. Maximum solvent productivity observed in R1 was 2.16 g/L.h at dilution rate 0.6 1/h corresponding to solvent yield of 0.36 g/g. In R2, the highest solvent productivity (2.07 g/L.h) was observed at a dilution rate 0.5 1/h with 0.31 g/g as solvent yield. Since the fermentation volume in R2 was double than R1, the dilution rate of 0.5 1/h in R2 corresponded to the dilution rate 1.0 1/h in R1, for which it showed a decrease in solvent production (0.77 g/L.h) and glucose consumption (10 %) in R1. Hence, dilution rate above 0.5 1/h in R2 was not practical to work with, and was decided as upper limit for this system.

The overall solvent production from both the reactors were calculated by considering the percent extraction from R1 and percent transfer of un-extracted solvent into R2 (Fig 3). Oleyl alcohol and decanol mixture was most effective with regard to butanol (90-92%) and ethanol (85-90%) extraction, but it was less effective with acetone (35-40%) and other acids (acetic acid and butyric acid) extraction. Butanol and ethanol are known to be strongly inhibitory to the organism, and hence their removal is obviously advantageous. The preferential extraction of solvents by extractant shows a minimum effect on butyric acid and acetic acid (reaction intermediates) extraction, enabling them to be available for further butanol production. Inability to extract these acids was favorable for higher solvent production, since it promotes the solventogenic phase and increases total solvent in the fermentation broth. Maximum total solvent production of 25.32 g/L (acetone, 5.93 g/L; butanol, 16.90 g/L; and ethanol, 2.48 g/L) was observed at dilution rate 0.05 1/h in first stage which was not possible to be achieved with single stage chemostat (15.98 g/L) because of butanol inhibition. Maximum solvent productivity (2.5 g/L.h) was also obtained at dilution rate 0.6 1/h in first stage and 0.3 1/h in second stage (overall dilution rate was 0.2 1/h) with the yield of 0.35 g/g and 60.34 % consumption of glucose. Maximum glucose utilization of 83.21 % at overall dilution rate 0.02 1/h was observed which was 28.83 % higher than single stage chemostat (54.38 %). Mutschlechner et al. (2000) reported maximum productivity of 0.27 g/L.h with overall solvent concentration of 15 g/L. Bahl et al. (1982) operated the first stage at dilution rate 0.125 1/h and second stage at dilution rate 0.04 1/h and

showed 87.5% utilization of the substrate but the solvent productivity was not observed to be high. Gapes et al. (1996) achieved an average overall solvent concentration of 9.27 g/L and productivity of 1.24 g/L.h with overall dilution rate 0.13 1/h.

Continuous ABE fermentation with *in situ* product removal system was operated successfully up to 30 days with higher solvent yield. In continuous ABE fermentation, fluctuations in ABE concentration (Fig 2 and Fig 3) were recorded in the effluent throughout the fermentation which is typical for such systems. This continuous culture over a prolonged period offers greater improvements in solvent productivity. However, this prolonged fermentation is difficult to achieve due to degeneration of *C. acetobutylicum* by the solvent produced. Also the fermentation is biphasic and maintenance of the culture exclusively in the solventogenic phase is difficult (Green, 2011). In continuous ABE fermentation, as the solventogenic *Clostridia* cells continue to undergo shifts between acidogenesis and solventogenesis, the bioreactor houses a mixture of actively dividing cells (acidogenic), non-dividing cells (solventogenic), sporulated cells, and dead cells. The oscillatory behavior of the ABE concentration in the bioreactor and effluent during the continuous ABE fermentation by *C. acetobutylicum* indicates that there are periods of time when the non-dividing cells (solventogenic) concentration is comparatively high or low in the bioreactor resulting in high or low ABE production (Lai and Traxler, 1994; Mulchandani and Volesky, 1994; Huang et al., 2004).

3.4. Effect of solvent extraction on glucose consumption

As shown in Fig. 4, solvent extraction generally enhanced glucose consumption with relatively higher consumption rates. Approximately 83% glucose was consumed when the extraction system of oleyl alcohol and decanol was integrated in between the two bioreactors in two-stage continuous fermentation which was much higher than by conventional fermentation (56%). Glucose consumption rate in single stage fermentation and in R1 of two-stage fermentation showed similar trend as shown in Fig. 4. Although R2 of two-stage fermentation showed somewhat unsteady trend, but it can be concluded to be nearly constant (30 to 40 %) for all the dilution rates, since the cells were already in glucose starved conditions with higher residence time. Overall glucose consumption was much higher than single stage fermentation with higher solvent productivity and higher solvent yield. As the dilution rate increased, the total solvent production and glucose utilization were found to be decreased (Fig. 4), which resulted in decrease in solvent yield and increase in solvent productivity. Dilution rate above 1.0 1/h in R1 resulted in decreased solvents production as well as glucose consumption and hence reached inhibitory threshold. Li et al., (2011) investigated continuous and fed-batch fermentation and showed that the time needed for passing from acidogenesis to solventogenesis was an intrinsic hindrance to higher butanol productivity. Therefore, an optimized dilution rate plays an important role in ABE fermentation. Lower concentrations of substrate shifts the system into acidogenic phase rather than solventogenic phase and lowers the solvent yield (Qureshi and Maddox, 1995). Hence consumption of substrate beyond 85% is not desirable for higher yields.

Although, the current system showed highest ABE productivity and yield with maximum glucose consumption, the use of pure glucose for solvent production would not be very cost effective. Hence, rigorous efforts on using other renewable resources are desired all over the world. In this context, the waste renewable sources such as cellulosic materials and algal biomass as raw material could be used in the present system which may enable the constant supply of liquid fuel to compensate world's energy demand. The highest solvent productivity target may enable further integration of three or more stage fermentation to maximally utilize the unconsumed sugars.

Conclusions

The integration of a liquid-liquid extraction (oleyl alcohol and decanol mixture) system in between the two stages of continuous ABE fermentation showed a progressive influence on solvent productivity, solvent yield and glucose utilization with *C. acetobutylicum* B 5313. *In situ* removal of solvents in present system can reduce the butanol toxicity towards the cells and enable high production of solvents (25.32 g/L) and solvent productivity (2.5 g/L.h) with maximum utilization of glucose. This process may allow the use of higher dilution rates of the feed and therefore reduce the costs of processing at industrial level.

References

- Assobhei, O., Kanouni, A.E., Ismaili, M., Loutfi, M., Petitdemange, H., 1998. Effect of acetic and butyric acids on the stability of solvent and spore formation by *Clostridium acetobutylicum* ATCC 824 during repeated subculturing. J. Ferment. Bioeng. 85(2),209–12.
- Bahl, H. W., Andersch, K., Braun and Gottschalk, G., 1982. Effect of pH and butyrate concentration on the production of acetone and butanol by *Clostridium acetobutylicum* grown in continuous culture. Appl. Microbiol. Biotechnol. 14, 17-20.
- Barton, W.E. Daugulis, A.J., 1992. Evaluation of solvents for extractive fermentation with *Clostridium acetobutylicum* and the use of poly(propylene glycol) 1200. Appl. Microbial. Biotechnol. 36, 632-639.
- Beefink, H.H., van Dillen, M., van den Heuvel, J.C., 1988. Formation of bacterial aggregates in a continuous-flow reactor: influence of carrier availability. Biotechnol. Lett. 10, 171–176.
- Davison, B.H. Thompson, J.E., 1993. Continuous direct solvent extraction of butanol in a fermenting fluidized bed reactor with immobilized *Clostridium acetobutylicum*. Appl. Biochem. Biotechnol. 39/40, 415-426.
- Evans, P.J. Wang, H.Y., 1988. Enhancement of butanol formation by *Clostridium acetobutylicum* in the presence of decanololeyl mixed extractants. Appl. Environ. Microbial. 54, 1662-667.

- Ezeji, T.C., Karcher, P.M., Qureshi, N., Blaschek, H.P., 2005. Improving performance of a gas stripping-based recovery system to remove butanol from *Clostridium beijerinckii* fermentation. *Bioprocess. Biosyst. Eng.* 27 (3), 207-214.
- Ezeji, T.C., Qureshi, N., Karcher, P., Blaschek, H.P., 2006. Butanol production from corn, in: Minteer, S.D. (Ed.), *Alcoholic Fuels: Fuels for Today and Tomorrow*. New York, NY: Taylor & Francis, pp. 99-122.
- Gapes, J.R., Nimcevic, D., Friedl, A., 1996. Long-term continuous cultivation of *Clostridium beijerinckii* in a two-stage chemostat with on-line solvent removal. *Appl. Environ. Microbiol.* 62(9), 3210–3219.
- Green, E.M., 2011. Fermentative production of butanol—the industrial perspective. *Curr. Opin. Biotechnol.* 22, 1–7
- Godin, C., Engasser J.M., 1989. Acid in the first stage is a determinant factor for the solvent production in the two-stage continuous fermentation of *Clostridium acetobutylicum*. *Biotechnol. Lett.* 11, 903–906.
- Grobbs, N.G., Eggink, G.F., Cuperus, F.P., Huizing, H.J., 1993. Production of acetone, butanol and ethanol (ABE) from potato wastes: fermentation with integrated membrane extraction. *Appl. Microbial. Biotechnol.* 39, 494-498.
- Huang, W.C., Ramey, D.E., Yang, S.T., 2004. Continuous production of butanol by *Clostridium acetobutylicum* immobilized in a fibrous bed bioreactor. *Appl. Biochem. Biotechnol.* 113–116, 887–898.
- Ishizaki, A., Michiwaki, S., Crabbe, E., Kobayashi, G., Sonomoto, K., Yoshino, S., 1999. Extractive acetone-butanol-ethanol fermentation using methylated crude palm oil as extractant in batch culture of *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564). *J. Biosci. Bioeng.* 87, 352–356.
- Job, C., Schertler, C., Staudenbauer, W.L., Blass, E. 1989. Selection of organic solvents for in situ extraction of productions from *Clostridium thermohydrosulfiricum* cultures. *Biotechnol. Tech.* 3, 315-320.
- Karcher, P., Ezeji T.C., Qureshi, N., Blaschek, H.P., 2005. Microbial production of butanol: product recovery by extraction, in: Satyanarayana, T., Johri, B.N., (Eds.), *Microbial Diversity: Current Perspectives and Potential Applications*. IK International Publishing House Pvt. Ltd. pp. 865-880.
- Koh, L.P., Ghazoul, J., 2008. Biofuels, biodiversity, and people: Understanding the conflicts and finding opportunities. *Biol. Conserve.* 141 (10), 2450–2460.
- Kumar, M., Gayen, K., 2011. Developments in biobutanol production: New insights. *Appl. Energy.* 88, 1999–2012

- Lai, M.C., Traxler, R.W., 1994. A coupled two-stage continuous fermentation for solvent production by *Clostridium acetobutylicum*. *Enzyme Microb. Technol.* 16,1021–1025.
- Lee, S.M., Cho, M.O., Park, C.H., Chung, Y.C., Kim, J.H., Sang, B.I., Um, Y., 2008. Continuous butanol production using suspended and immobilized *Clostridium beijerinckii* NCIMB 8052 with supplementary butyrate. *Energy Fuels*, 22(5), 3459–3464.
- Li, S.Y., Srivastava, R., Suib, S.L., Li, Y., Parnas, R.S., 2011. Performance of batch, fed-batch, and continuous A-B-E fermentation with pH-control. *Bioresour.Technol.* 102 (5), 4241-4250
- Maddox, I.S., 1989. The acetone-butanol-ethanol fermentation: recent progress in technology. *Biotechnol. Genet. Eng. Rev.* 7, 189–220.
- Matta-el-Ammouri, G., Janati-Idrissi R., Junelles, A.M., Petitdemange, H., Gay, R., 1987. Effects of butyric and acetic acids on acetone butanol formation by *Clostridium acetobutylicum*. *Biochim.* 69, 109-115.
- Monot, F., Martin, J.R., Petitdemange, H., Gay, R., 1982. Acetone and butanol production by *Clostridium acetobutylicum* in a synthetic medium. *Appl. Environ. Microbial.* 44(6), 1318-1324.
- Mulchandani, A., Volesky, B., 1994. Production of acetone–butanol–ethanol by *Clostridium acetobutylicum* using a spin filter perfusion bioreactor. *J. Biotechnol* 34, 51–60.
- Mutschlechner, O., Swoboda, H., Gapes, J.R., 2000. 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.* 2(1), 101-105.
- Qureshi, N., Maddox, I.S., 1995. Continuous production of acetone-butanol-ethanol using immobilized cells of *Clostridium acetobutylicum* and integration with product removal by liquid liquid extraction. *J. Ferment. Bioeng.* 80, 185-189.
- Qureshi, N., Maddox, I.S., 2005. Reduction of butanol inhibition by perstraction: utilization of concentrated lactose/whey permeate by *Clostridium acetobutylicum* to enhance butanol fermentation economics. *Food Bioprod Process*, 83, 43–52.
- Qureshi, N., Schripsema, J., Lienhardt, J., Blaschek, H.P., 2000. Continuous solvent production by *Clostridium beijerinckii* BA101 immobilized by adsorption onto brick. *World J. Microbiol. Biotechnol.* 16(4), 377–382.

- Ramos, M.J., Fernández, C.M., Casas, A., Rodríguez, L., Pérez, A., 2009. Influence of fatty acid composition of raw materials on biodiesel properties. *Bioresour. Technol.* 100, 261–268.
- Shukla, R., Kang, W., Sirkar, K.K. 1988. Toxicity of organic solvents to *Clostridium acetobutylicum* for extractive ABE fermentation. *Appl. Biochem. Biotechnol.* 18, 315–324.
- Survase, S.A., Sklavounos, E., Jurgens, G., van Heiningen, A., Granström, T., 2011a. Continuous acetone–butanol–ethanol fermentation using SO₂–ethanol–water spent liquor from spruce. *Bioresour. Technol.* 102, 10996–11002.
- Survase, S.A., Jurgens, G., van Heiningen, A., Granström, T., 2011b. Continuous production of isopropanol and butanol using *Clostridium beijerinckii* DSM 6423. *Appl. Microbiol. Biotechnol.* 91(5), 1305–1313.
- Zhang, Y., Ma, Y., Yang, F., Zhang, C., 2009. Continuous acetone–butanol–ethanol production by corn stalk immobilized cells. *J. Ind. Microbiol. Biotechnol.* 36, 1117–1121.
- Zverlov, V.V., Berezina, O., Velikodvorskaya, G.A., Schwarz, W.H., 2006. Bacterial acetone and butanol production by industrial fermentation in the Soviet Union: use of hydrolyzed agricultural waste for biorefinery. *Appl. Microbiol. Biotechnol.* 71, 587–597.

Fig. 1. Schematic diagram of optimum continuous reactor with sugarcane baggase as a cell holding material for production of solvents (butanol and isopropanol). 1=weighing balance, 2=Feed tank, 3=bioreactor 1 (R1), 4=cells immobilized in R1, 5=Sample port from R1, 6=extraction vessel, 7=broth and extractant mixture, 8=separation vessel, 9=organic phase containing solvent and acids, 10=aqueous phase, 11=outlet for extracted solvent/inlet for fresh extractant, 12=sample port from separation vessel, 13=bioreactor 2(R2), 14=cells immobilized in R2, 15=sample port from R2, 16=collection vessel

Fig. 2. Product formation and substrate consumption in single-stage continuous culture of *C. acetobutylicum* B 5313

Fig. 3. Overall Product formation and substrate consumption in two-stage continuous culture of *C. acetobutylicum* B 5313 integrated with liquid-liquid extraction system

Fig. 4. Effect of dilution rate on glucose consumption with single stage continuous fermentation and two-stage continuous extractive fermentation of *C. acetobutylicum* B 5313