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The two stage immobilized column reactor with an integrated solvent recovery module for enhanced ABE production

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Abstract

The production of acetone, butanol, and ethanol (ABE) by fermentation is a process that had been used by industries for decades. Two stage immobilized column reactor system integrated with liquid–liquid extraction was used with immobilized *Clostridium acetobutylicum* DSM 792, to enhance the ABE productivity and yield. The sugar mixture (glucose, mannose, galactose, arabinose, and xylose) representative to the lignocellulose hydrolysates was used as a substrate for continuous ABE production. Maximum total ABE solvent concentration of 20.30 g.L⁻¹ was achieved at a dilution rate (D) of 0.2 h⁻¹, with the sugar mixture as a substrate. The maximum solvent productivity (10.85 g.L⁻¹h⁻¹) and the solvent yield (0.38 g.g⁻¹) were obtained at a dilution rate of 1.0 h⁻¹. The maximum sugar mixture utilization rate was achieved with the present set up which is difficult to reach in a single stage chemostat. The system was operated for 48 days without any technical problems.

Keywords: *n*-Butanol, *Clostridium acetobutylicum*, Immobilization, ABE solvents, Sugar mixture.

1. Introduction

In order to meet the expected increasing demand for biofuels and biochemicals, and to diversify the feedstock and product portfolio of biorefineries, there is an extensive need to find additional suitable biomass sources, which do not rely on using large amounts of agricultural land. According to the recent report, *n*-butanol is currently produced on a scale of 5–6 million tons per year, with a worldwide market sale of US\$7–8.4 billion (Cheng et al., 2012). Moreover, the market demand is anticipated to increase dramatically, if *n*-butanol can be produced more cost-effective way (Cheng et al., 2012). The use of inexpensive and renewable feedstock has gained some attention recently to reduce biobutanol production cost, such as lignocellulosic materials (agricultural waste, paper waste, wood chips, *etc.*) which are abundant and sustainable. These agricultural biomass sources cost much less (US\$24–75/ton) than the traditional substrates (Qureshi et al., 2013). Butanol is an ideal alternative fuel because of its high energy content, low volatility, and characteristics of being less hygroscopic and less corrosive. It can also substitute gasoline without the need to alter any current vehicle or engine technologies (Campos-Fernández et al., 2012).

Some *Clostridium* species are able to produce acetone, butanol and ethanol (ABE) by anaerobic fermentation from a wide variety of sugars both hexoses and pentoses. These species are able to utilize sugars from variety of substrates, including (lignocellulosic) hydrolysates derived from plant biomass. This process is commonly referred to as ABE fermentation (Survase et al., 2011). Butanol producing cultures can simultaneously utilize hexose and pentose sugars which are present in the biomass hydrolysates (Ezeji and Blaschek, 2008). *n*-Butanol is the major chemical end product in ABE fermentation, and the fermentation reaction requires cofactor balance in the metabolism especially to the oxidation-reduction cofactors (such as NADH) to proceed. A high carbohydrate concentration (e.g., glucose), which provides both the necessary carbon source and the energy for the microbial species, is critical to ensure the successful ABE fermentation (Karakashev et al., 2007).

Butanol is known to inhibit the metabolic pathways of butanol-producing bacteria leading to feedback repression of butanol production. The toxicity is mainly caused by its hydrophobic nature that increases the fluidity of the cell membrane. As a result, the function of the cell membrane as a controlling barrier between cell interior and exterior is affected. Additionally, the transmembrane pH gradient is destroyed resulting in energy shortage (Garcia et al., 2011). However, comparative quantification of butanol inhibition upon myriads of biochemical characteristics of these bacteria is still unexplored due to its complicated reaction network for butanol formation (Garcia et al., 2011). Butanol production further remained unpredictable because of other unwanted products (acetic acid) and lack of adequate inhibition figures (Chen et al., 2012). Among various Clostridia, *C. acetobutylicum* and *C. beijerinckii* are recognized as high butanol producers (Kumar and Gayen, 2011). However, some of them face the challenges which include acid crash due to excess acid production before the solventogenic phase, degeneration of solvent producing capability due to repeated sub-culturing, sporulation during solvent production, and a solvent intolerance (Li et al., 2011). To overcome these obstacles, metabolic and genetic engineering tactics are constantly employed by many research groups (Dunlop, 2011; Lehmann and Lutke-Eversloh, 2011; Yu et al., 2011). Apart from the metabolic studies, the development of a simple and an economic bioprocess is also desirable to maximize the butanol production as explained herein this study.

ABE productivity in batch reactors is often low due to downtime, long lag phase and a product inhibition. Gu et al. (2009) found an increase in the solvent productivity by *C. acetobutylicum* with the addition of ammonium acetate using cassava medium. Continuous fermentations have an advantage of a shorter downtime, automatic operation tends to be simpler

and usually they exhibit higher productivity than in batch fermentations (Jin et al., 2011). A single continuous fermentation reactor has process challenges on an industrial scale *viz.* incomplete substrate consumption, low solvent productivity and stability (Lee et al., 2008). Commercial plants in Russia and China were operated in a continuous mode using a series of reactors in parallel to improve the overall productivity (Ni and Sun, 2009; Green, 2011). A chemostat with *in-situ* butanol removal from the fermentation system by adsorption, liquid–liquid extraction, pervaporation and with gas stripping have been used previously to mitigate the butanol inhibition and to improve the productivity (Bankar et al., 2012; Qureshi et al., 2008). However, a common problem of these techniques is their low efficiency in recovering the butanol with a high concentration even though the productivity of the fermentation system could be improved effectively.

The use of two-stage continuous solvent-producing cultures with the immobilized biomass or biomass-retention achieves a much higher solvent productivities due to the possibility of sustaining higher dilution rates. The immobilization by a passive adhesion to the surface of a carrier is more preferred due to the limited problems related to diffusion. In order to use this technique effectively, in-depth understanding of the surface properties of microbial cells as well as the fibrous matrices is essential. Currently, agricultural immobilization materials which are recycled and reused are more common in use (Survase et al., 2012).

With the experimental set-up presented here, integration of an *in-situ* solvent recovery module in between the two immobilization columns was studied to improve the substrate consumption and to reduce the solvent toxicity. The effect of various dilution rates on the productivity and the yield was evaluated in this study. *C. acetobutylicum* DSM 792 was explored as a test organism for immobilization. Recyclable and biodegradable wood pulp fibers were used as an immobilization material for the continuous production of ABE. The performance of the immobilized cell reactor was investigated for the production of ABE solvents using sugar mixture as substrates.

2. Materials and methods

2.1 Materials

D-Glucose and D-xylose were purchased from VWR International, Finland. Yeast extract and tryptone were purchased from Lab M Ltd, UK. D-Mannose, D-galactose, and L-arabinose were purchased from Danisco, Finland. p-amino benzoic acid, MgSO₄, FeSO₄, and MnSO₄ were obtained from Fluka, Switzerland. L-Cysteine hydrochloride, thiamin and biotin were purchased from Sigma Aldrich, USA. K₂HPO₄, KH₂PO₄, ammonium acetate, and reinforced clostridia medium (RCM) were obtained from Merck, Germany. Oleyl alcohol and decanol were obtained from SAFC, and Sigma Aldrich, USA respectively. NaOH, NaCl and HCl were obtained from J.T. Baker, Holland. All the chemicals were of an analytical grade. The wood pulp fibers were obtained by SO₂-water-ethanol (SEW) fractionation process from the Department of Forest Products Technology, School of Chemical Technology Aalto University, Finland.

2.2 Microorganism and medium

C. acetobutylicum DSM 792 was obtained from DSMZ, Germany (German Collection of Microorganisms and Cell Cultures). The culture was maintained on a reinforced clostridia medium (RCM) as explained earlier (Bankar et al., 2012). The production medium reported by Tripathi et al.(2010) was modified to use a sugar mixture as a carbon source containing glucose, mannose, arabinose, galactose, and xylose as a sole replacement to glucose (60 gL⁻¹). Other medium components were (in gL⁻¹), magnesium sulfate 0.2, sodium chloride 0.01, manganese sulfate 0.01, iron sulfate 0.01, potassium dihydrogen phosphate 0.5, potassium hydrogen phosphate 0.5,

ammonium acetate 2.2, biotin 0.01, thiamin 0.1, and *p*-aminobenzoic acid 0.1. Sugar mixture contained ($\text{g}\cdot\text{L}^{-1}$) glucose 32.73, mannose 14.78, arabinose 2.14, galactose 3.95, and xylose 6.41 to get total sugars to be $60 \text{ g}\cdot\text{L}^{-1}$. If necessary, the medium was adjusted to pH 6.5 with HCl. After preparation, the medium was purged with nitrogen and autoclaved at 203.4 kPa (121 °C) for 20 min and cooled.

2.3 Preparation of column reactors and operation

Our previous studies have indicated that the higher reactor productivities can be achieved by using immobilized cell reactors or immobilized cell columns which in turn reduce the cost of production (Bankar et al., 2012, Survase et al., 2012). Present study employed the use of the two stage immobilized column reactors with an integrated liquid-liquid extraction module to enhance the solvent productivity and yield. The liquid-liquid extraction integrated solvent removal system was adopted from our previous study (Bankar et al., 2012). Different extraction parameters like oleyl alcohol to decanol ratio, broth to extractant ratio, extraction time, distribution coefficient, extraction efficiency and butanol selectivity were optimized previously (Bankar et al., 2012) and applied into the present system. The experimental setup with the two immobilized columns in series and an integrated liquid-liquid extraction module for *in situ* solvent removal is shown in Fig. 1.

The column reactors were prepared as reported by Survase et al. (2012). The wood pulp fibers were rolled in a nylon mesh and inserted into the jacketed glass columns. Column 1 (stage 1) and column 2 (stage 2) consisted of 50 ml and 75 ml void volumes, respectively. Both the columns were decontaminated with the ethanol (70%) for 24 h. The inoculum was prepared as described previously using activated spore culture in air-tight, anaerobic glass bottles and grown for 20 h at 37 °C. Ethanol in the column reactors was replaced with the production medium and it was inoculated with the 20 h old highly motile cells of *C. acetobutylicum* DSM 792. Fermentation was allowed to proceed in the batch mode for 24 h with the re-circulation through both the columns (without an integration of the extractor and the settling vessel) to assist the cell growth and immobilization. After the cells were immobilized, the fermentation feed medium was continuously pumped into the stage 1 column at different dilution rates.

The extraction module and the settling tank consisted of two glass jacketed bioreactors with a total volume of 1 L. They contained the mixture of oleyl alcohol and decanol (4:1) sterilized by autoclaving at 121° C for 20 min. The assembly was arranged sequentially and designated as stage 1 (3), extractor (6), settling vessel (7) and stage 2 (12) (Fig. 1). Temperature of the columns, extractor and settling vessel was maintained at 37 °C by continuously circulating water through the jacket. Extraction vessel was stirred at 400 rpm while the settling tank was not agitated to allow the separation of organic and aqueous phases.

Fresh medium was continuously introduced into the stage 1 from the bottom of the column with a peristaltic pump. The dilution rate of stage 1 was varied between 0.25 and 3.0 h^{-1} which resulted in the dilution rate range of $0.167\text{--}2.0 \text{ h}^{-1}$ in stage 2. Dilution rate was calculated based on the void volume of the column. The steady state was confirmed by the constant product values at specific dilution rates. Samples for product analysis were taken after five working volume change for three consecutive days.

Culture broth from the first column reactor and the extractant were mixed by continuous agitation. The volume of the extractor was maintained at 400 mL by maintaining the flow rate to the settling vessel and the back flow of the extractant from the settling vessel to the extractor (Fig. 1). The mixture was allowed to separate in the settling vessel (7) into organic and aqueous phases. The latter was pumped to the stage 2 column reactor where further fermentation was carried out by immobilized cells. The organic phase from the settling vessel was recycled back

into the extraction vessel to maintain the volume of 400 mL. All the physiological parameters were kept the same for both the columns except the column volume (50 ml in the first stage and 75 ml in the second stage). The fresh extractant was introduced in extraction vessel (Fig. 1) after 10 days during lower dilution ($0.1 \text{ h}^{-1} - 0.6 \text{ h}^{-1}$) rates and after 15 days during higher dilution rates ($0.8 \text{ h}^{-1} - 1.2 \text{ h}^{-1}$) to keep extractant unsaturated with the solvents. The input substrate concentration in the stage 1 was obviously higher than in the stage 2. This resulted in lower growth in the second stage. Lower specific consumption rate of monosaccharides justified the higher volume and longer residence time in the second stage to get the maximum substrate utilization.

2.4 Determination of substrates and products

Solvents and acids were quantified by using gas chromatography as described by Survase et al. (2011). Gas chromatography (Hewlett Packard series 6890) equipped with a flame ionization detector and DB-WAXetr capillary column ($30 \text{ m} \times 0.32 \text{ mm} \times 1 \mu\text{m}$) was used. Injector temperature was $200 \text{ }^\circ\text{C}$ and the detector temperature was $250 \text{ }^\circ\text{C}$. The injector volume was $10 \mu\text{L}$. Glucose, mannose, arabinose, galactose, and xylose were determined by high performance liquid chromatography (Bio-Rad Laboratories, Richmond, CA, USA) equipped with an Inores S 259-H column (Inovex, Vienna, Austria) packed with Inores cation exchanger (particle size, 9 mm). Refractive index detector (model 1755; Bio-Rad) was used for the quantification while cellobiose (Roth, Karlsruhe, Germany) solution was used as an internal standard. Column was heated at $70 \text{ }^\circ\text{C}$, and the eluent ($0.01 \text{ M H}_2\text{SO}_4$) was circulated with a flow rate of $0.60 \text{ mL} \cdot \text{min}^{-1}$.

2.5 Calculation of bioprocess parameters

Calculations for dilution rate, solvent yield and solvent productivity have been reported earlier (Bankar et al., 2012, Survase et al., 2012). Overall dilution rate (h^{-1}) was calculated by dividing the feed flow rate with the total volume of the columns. The leaked cells from the immobilized stage 1 column were consumed in the settling tank with the concomitant acid and solvent production. Since, the solvent production and the substrate consumption values in settling tank were very low as compared to stage 1 and stage 2, the working volume of settling tank was not considered in the solvent productivity calculations. The solvent production in the stage 1 column, settling vessel and in the stage 2 column were analyzed separately to cumulate all the three readings. The extraction efficiency for solvents (in %, acetone 35; butanol 90; ethanol 85) and for acids (in %, acetic acid < 5 ; butyric acid < 5) was considered while calculating the actual solvent and acid production in the stage 2 column (Bankar et al., 2012). Similarly, the substrate utilizations (%) were also determined individually in stage 1, settling vessel and in stage 2 column. The overall solvent productivity and the solvent yields were calculated by considering the amount of un-extracted solvents and non-utilized substrates transferred from the settling vessel into the stage 2.

3. Results and discussion

Continuous cultures with an immobilized biomass or biomass retention are reported to give high solvent productivities due to high dilution rates (Green, 2011). However, while getting higher productivities, the loss of substrates has been observed due to the short residence times, which make the process uneconomical (Survase et al., 2012). If the process is operated at low dilution rates, the problem of solvent toxicity has to overcome. The attempt to handle both of these problems using an optimized system where reactors were used in a series along with an integrated solvent removal module to reduce the toxicity of the solvents is presented here.

To overcome the toxicity of the solvents especially by *n*-butanol, it was of an interest to investigate the highly selective water-immiscible extractants to remove *in-situ* acetone, *n*-butanol and ethanol. *In-situ* removal of the inhibitory product *n*-butanol during fermentation has been

shown to increase the solvent titres and yields (Bankar et al., 2012, Survase et al., 2012). The significant decrease in distillation costs with these extractants is due to lower heat capacities and higher boiling points of the extractants (e.g. 2.32 kJ.kg⁻¹.K⁻¹ vs. 4.18 kJ.kg⁻¹.K⁻¹ for oleyl alcohol and water, respectively). Also, decrease in water use and reactor size contributes to decrease the overall cost of the product (Roffler et al., 1987). The *in-situ* removal of ABE from the aqueous phase by a high boiling point extractant such as oleyl alcohol, decanol and glyceryl tributyrates decreases the energy requirements for the product distillation and allows the integration of biological and catalytic processes (Anbarasan et al., 2012, Kraemer et al., 2011). The liquid–liquid extraction has been reported to extract butanol from the fermentation broth without removing the substrates, water or nutrients (Davison and Thompson, 1993). Previous studies in our laboratory showed that the mixture of oleyl alcohol and decanol (4:1) had the best extraction efficiency without any toxicity towards the cells (Bankar et al., 2012).

3.1 Two-stage ABE fermentation with integrated liquid–liquid extraction

Wood pulp as an immobilization material was used in the present study for the continuous production of ABE solvents. Wood based biorefinery with an integrated pulp; hemicellulose and lignin streams with continuous biofuels production would be beneficial in terms of the cost effectiveness of the process.

The reactor efficiency was investigated with respect to the dilution rate to attain the maximum solvent productivity, maximum substrate utilization, and solvent concentration in the effluent stream. A sugar mixture (g.L⁻¹) (glucose 32.73, mannose 14.78, arabinose 2.14, galactose 3.95, and xylose 6.41) was used as a substrate representing a lignocellulose hydrolysate. Stage 1 and stage 2 immobilized columns were operated with an integrated product recovery mode using oleyl alcohol and decanol (4:1) as extractants at different dilution rates. The overall dilution rates and products formed in the bioreactors are shown in Fig. 2. The overall solvent production from both the reactors was calculated by considering the percent extraction from the stage 1 and percent of non-extracted solvents transfer into the stage 2. The mixture of oleyl alcohol and decanol was effective for butanol and ethanol extraction, but it was less effective for extracting the acetone and acids (acetic acid and butyric acid). The removal of butanol and ethanol is valuable since both of these are known to be strong inhibitors to the organisms (Ni and Sun, 2009, Green, 2011). The minimal effect on extraction of butyric acid and acetic acid (reaction intermediates) enables them to be available for further reassimilation into solvents production (Bankar et al., 2012; Cho et al., 2012).

The maximum overall total solvent production of 20.30 g.L⁻¹ (acetone, 4.93 g.L⁻¹; butanol, 13.58 g.L⁻¹; and ethanol, 1.80 g.L⁻¹) was observed at a dilution rate of 0.2 h⁻¹. The production of acetic and butyric acid corresponded to the total solvents produced which were decreased with an increase in the dilution rate. This confirmed the role of acids reassimilation for the production of solvents (Fig. 2). *C. acetobutylicum* DSM 792 is known to tolerate very high amounts of the total acids (upto 11.7 g.L⁻¹) (Cho et al., 2012) as compared to the total acids produced in the present system (6.3 g.L⁻¹). Hence, by observing the results for sugar utilization, solvent productivity and solvent yield, a hypothesis can be made that the acidic crash cannot be seen in the present system. The highest solvent productivity (10.85 g.L⁻¹.h⁻¹) obtained at a dilution rate of 1.0 h⁻¹ with the yield of 0.38 g.g⁻¹ (Fig. 3) made this technology feasible for the cost effective production of solvents from lignocellulosic sugar mixtures. As the dilution rate was increased from 0.1 to 1.2 h⁻¹ the solvent productivity was increased accordingly up to 1.0 h⁻¹. The results of this study were promising and better than our previous studies with *C. acetobutylicum* (Bankar et al., 2012, Survase et al., 2012). Table 1 details the comparative representation of the results from the previous study and those presented here, which indicates the advantages of the

present system in all aspects. Although, the ABE production in a single stage column (Survase et al., 2012) and the stage 1 of the present study were similar, the overall solvent production in the present study was significantly better than in the single stage study. Previously, the ABE production in the two stage immobilized reactor with an *in-situ* recovery module showed lower solvent productivity ($2.5 \text{ g.L}^{-1}\text{h}^{-1}$) as compared to present study ($10.85 \text{ g.L}^{-1}\text{h}^{-1}$) (Table 1) (Bankar et al., 2012, Survase et al., 2012). Moreover, the use of pure glucose during the old process made it expensive and hence the present technique with the cost effective sugar mixture substrate overcame the issues related to the process economy. Total solvents, solvent productivity and solvent yields were found to be higher when process economy of the present technique was taken into consideration (Table 1).

Table 2 reports the utilization of different sugars from the sugar mixture at varied dilution rates. Glucose was found to be the most preferred substrate followed by arabinose, mannose, and xylose. Galactose was the least preferred carbon source. The complete utilization of xylose, arabinose and galactose was found to be incomplete with the single stage column reactor (Survase et al., 2012), unlike with the two stage column reactors integrated with the solvent recovery module. The maximum total sugar utilization of 93.72% at an overall dilution rate of 0.2 h^{-1} was observed which was 12 % higher than that of single stage reactor (Table 1). Recently, various researchers have reported different values of ABE production which are presented in Table 3. Sugars other than glucose in the mixture can be utilized effectively if sufficient amount of glucose is present. Ezeji and Blaschek (2008) reported that, although all the sugar components of a sugar mixture are utilized concurrently by clostridia, glucose is the most preferred among the mixture components. In addition, they reported that the availability of excess fermentable sugars in the broth is necessary for both the onset and the maintenance of solvent production. Otherwise, the fermentation will become acidogenic leading to the premature termination of the process.

The present ABE continuous column reactors with the *in-situ* product removal system were operated successfully for up to 48 days (1152 h) with the higher solvent productivity and yield (Fig. 4). Total 91.5 L feed medium was passed through both the columns with the continuous regeneration of the cells without a heavy leakage from the columns. This continuous culture over a prolonged period offer improvements into the solvent productivity. Green (2011) concluded that this kind of prolonged fermentation is difficult to achieve due to degeneration of *C. acetobutylicum* by the produced solvent. Also the fermentation is biphasic and maintenance of the culture exclusively in the solventogenic phase was claimed to be difficult (Green, 2011). In a continuous ABE fermentation the solventogenic *Clostridia* cells continue to undergo the shifts between acidogenic and solventogenic phases. The bioreactor houses a mixture of actively dividing cells (acidogenic), non-dividing cells (solventogenic), sporulating and dying cells. The oscillatory behavior of the ABE concentration in the bioreactor and in the effluent stream during the continuous ABE fermentation by *C. acetobutylicum* indicates that there are periods of time when the concentration of the non-dividing cells (solventogenic) is comparatively high or low in the bioreactor resulting in high or low ABE production (Huang et al., 2004).

Several ABE fermentation studies have been reported in the literature, which feature batch and/or continuous cultures with either freely suspended or immobilized cells. These cultures were run successfully for prolonged periods of time (Table 3). These studies differed from our study in several aspects. The major one being the productivity with higher dilution rate which we report here to be highest from the lignocellulosic sugar mixture as compared to the other reports. By considering the solvent productivity, yield, sugar utilization (pentose and hexose) and the stability of the process, the present system appear to be the best suited for the industrial production.

Qureshi et al. (2000) used the clay brick as a carrier material in an immobilized column with hyper-butanol-producing *C. beijerinckii* BA101, and reported the highest solvent productivity to date to be 15.8 g.L⁻¹h⁻¹ at the dilution rate of 2 h⁻¹. However, the use of pure glucose and the lower substrate utilization were hindrances in the process economy, which were overcome in the present study (Table 1 and 2). Although further improvements will be required for commercial implementation; these results indicate that the two stage immobilized column reactors with an *in-situ* extraction could significantly contribute into the ABE production research.

4. Conclusions

Immobilized cells on wood pulp provide the long run system operation (48 days) with the high solvent productivity (10.85 g.L⁻¹h⁻¹) and yield (0.38 g.g⁻¹). Substantial amount of sugar mixture utilization (93.72 %) (both pentose and hexose sugars) with the wild-type strain suggests an industrial feasibility of this process. The immobilized column reactor with wood pulp can become an efficient technology to be integrated with the existing pulp mills to convert them into wood based bio-refineries. The establishment of a long term stable conversion process which is mentioned in the present study is a benchmark for further research.

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Figure captions

Fig. 1 Schematic diagram of the two stage chemostat with an integrated solvent recovery module

Fig. 2 The effect of dilution rate on overall solvent and acid formation in the two-stage continuous culture of *C. acetobutylicum* DSM 792 integrated with the liquid–liquid extraction system

Fig. 3 Overall solvent productivity, solvent yield, total sugars consumed and total solvents produced in the two-stage continuous culture of *C. acetobutylicum* DSM 792 integrated with the liquid–liquid extraction system

Fig. 4 Time course of the fermentation with respect to dilution rate showing the production of the solvents and solvent productivity in the two-stage continuous culture with the integrated extraction system.

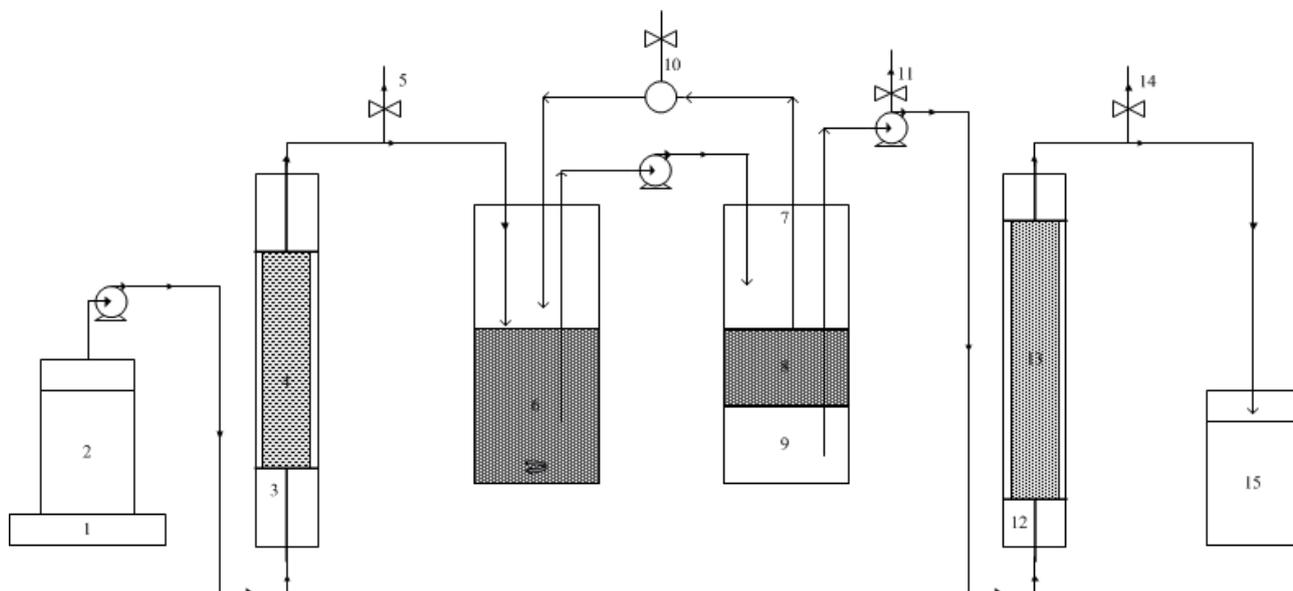


Fig. 1 Schematic diagram of the two stage chemostat with an integrated solvent recovery module

1 = weighing balance, 2 = feed tank, 3 = first column, 4 = cells immobilized in the first column, 5 = sample port from the column 1, 6 = extraction vessel, 7 = separation vessel, 8 = organic phase containing solvent, 9 = aqueous phase, 10 = outlet for extracted solvent/inlet for fresh extractant, 11 = sample port from the settling vessel, 12 = second column, 13 = cells immobilized in the second column, 14 = sample port from the column 2, 15 = collection vessel.

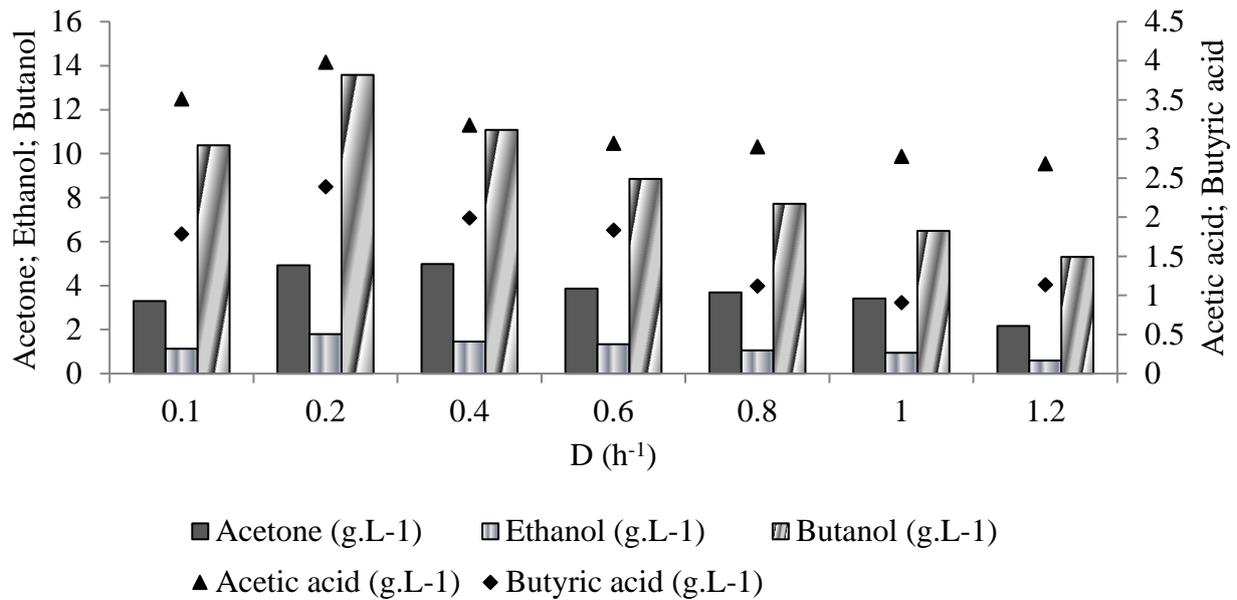
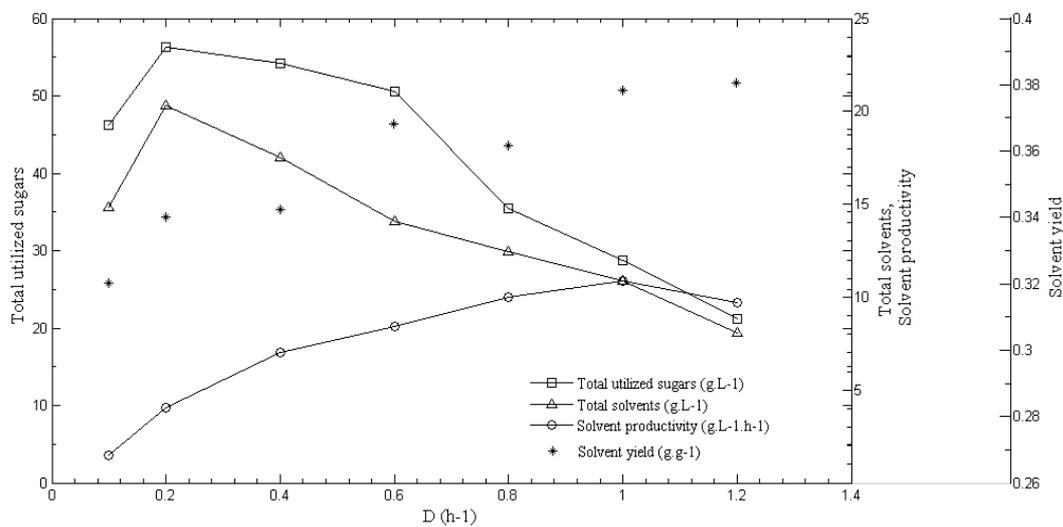
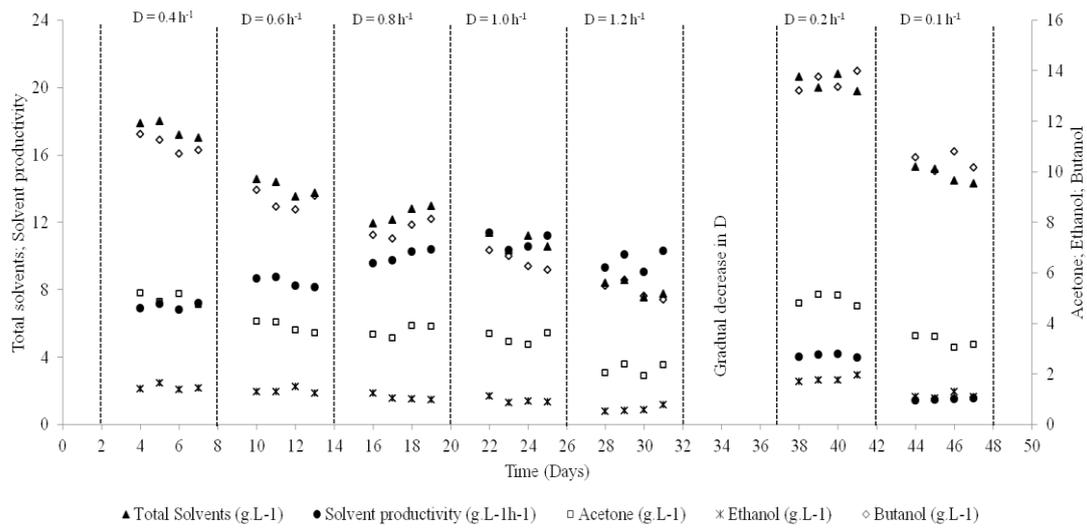


Fig. 2 The effect of dilution rate on overall solvent and acid formation in the two-stage continuous culture of *C. acetobutylicum* DSM 792 integrated with the liquid–liquid extraction system



*Total sugars were corresponded to 58 g.L⁻¹ after autoclave

Fig. 3 Overall solvent productivity, solvent yield, total sugars consumed and total solvents produced in the two-stage continuous culture of *C. acetobutylicum* DSM 792 integrated with the liquid–liquid extraction system



*Dilution rate (D) was decreased gradually (with one unit) from day 32 to 37 to study the lower dilution rates.

Fig. 4 Time course of the fermentation with respect to dilution rate showing the production of the solvents and solvent productivity in the two-stage continuous culture with the integrated extraction system.

Table 1 Summary of our previous studies for continuous column reactor performance with the cell immobilization technique

Parameter ^a	Single stage immobilized column (Survase et al., 2012)	Two stage immobilized reactor with <i>in situ</i> recovery (Bankar et al., 2012)	Two stage with <i>in situ</i> recovery	Immobilized column	Overall ^d
			First stage	Second stage ^c	
Temperature (°C)	37	37	37	37	37
Dilution rate (h ⁻¹)	0.5	0.2	0.5	0.33	0.2
Total solvents (g.L ⁻¹)	12.63	25.32	12.41	6.31	20.30
Acetone (g.L ⁻¹)	4.12	5.93	4.04	0.84	4.93
Butanol (g.L ⁻¹)	7.24	16.90	7.57	4.81	13.58
Ethanol (g.L ⁻¹)	1.27	2.48	0.80	0.66	1.80
Acetic acid (g.L ⁻¹)	0.6	2.87	2.12	1.52	3.98
Butyric acid (g.L ⁻¹)	1.28	2.54	1.36	0.87	2.39
Highest Solvent productivity (g.L ⁻¹ h ⁻¹)	12.14	2.5	9.58	9.56	10.85
Highest Solvent yield ^b (g.g ⁻¹)	0.33	0.35	0.40	0.39	0.38
Total utilized sugars (%)	82.00	83.21	80.72	69.32	93.72
Glucose utilized %	100	83.21	100	No glucose	100
Mannose utilized %	92.49	-	69.99	19.64	92.56
Xylose utilized %	66.8	-	51.88	33.44	84.01
Arabinose utilized %	83.5	-	72.25	100	100
Galactose utilized %	33.17	-	32.73	58.90	64.65

^aAverage values during steady-state of fermentation.

^bSolvent yield was calculated from total sugar concentrations in the feed medium.

^cCalculations were done by considering amount of solvents and sugars transferred from the first stage column and the extractor

^dOverall solvent and sugar consumption calculations considered the non-extracted solvents and the solvents produced in settling tank

Table 2 Overall utilization of sugars during the continuous production of ABE solvents using the immobilized two stage column reactors by *C. acetobutylicum* DSM 792 with the sugar mixture as a substrate.

D (h ⁻¹)	Arabinose (g.L ⁻¹)	Galactose (g.L ⁻¹)	Glucose (g.L ⁻¹)	Xylose (g.L ⁻¹)	Mannose (g.L ⁻¹)	Total sugars (g.L ⁻¹)	Total sugar utilization (%)
Feed	2.14	3.95	32.73	6.41	14.78	58.00	0.00
0.25	1.63	0.30	32.73	1.17	10.40	44.22	76.24
0.50	2.14	2.43	32.73	5.38	13.68	54.36	93.72
1.00	2.08	1.80	32.73	5.40	12.27	52.26	90.11
1.50	1.88	1.00	32.73	3.19	11.76	48.56	83.72
2.00	0.83	0.26	28.65	1.32	4.40	33.45	57.67
2.50	0.20	0.12	25.49	0.81	2.06	26.68	46.00
3.00	0.00	0.04	15.44	0.51	1.32	15.21	26.22

1 **Table 3** Summary of ABE production in different physiological conditions.

System	Strain	Total sugar utilization (g.L ⁻¹)	D (h ⁻¹)	ABE productivity (g.L ⁻¹ h ⁻¹)	Total solvent yield (g.g ⁻¹)	Highest <i>n</i> –butanol (g.L ⁻¹)	Stability	Reference
Continuous two stage <i>insitu</i> product removal	<i>C. saccharoperbutylacetonicum</i> strain N1-4	Glucose: 93%	0.025	-	butanol: 0.386	4.0	1008 h	(Richter et al., 2012)
Two stage immobilized batch, <i>insitu</i> product removal	<i>C. acetobutylicum</i> JB200	Glucose 100%	-	0.66	0.40	19.8	48 h	(Xue et al., 2012)
Two-stage batch, controlled-pH strategy,	<i>C. acetobutylicum</i> XY16		-	0.63	0.32	11.5	32 h	(Guo et al., 2012)
Continuous reactor coupled to a repeated fed-batch reactor	<i>C. acetobutylicum</i> ATCC 824	Glucose 100%	0.1	1.47	0.39	12.0	190 h	(Setlhaku et al., 2012)
Batch/ fed batch, in situ butanol removal using biodiesel extraction	<i>C. acetobutylicum</i> BCRC 10639	Glucose 100%	-	0.295	0.31	31.44	96 h	(Yen and Wang, 2012)
Integrated product recovery by cyclic vacuum	<i>C. beijerinckii</i> ATCC 51743	Glucose 100%		0.37	0.34	44.2 (in condensate)	55 h	(Mariano et al., 2012)
Coupled continuous two-stage fermentation	<i>C. acetobutylicum</i>	-	0.06	0.6	-		750 h	(Lai and Traxler, 1994)
Two-stage continuous cultivation	<i>C. beijerinckii</i> NRRL B592	Glucose 98 %	0.018	0.27	0.25	9.1	1600 h	(Mutschlechner et al., 2000)
Continuous ABE production using degermed corn	<i>C. beijerinckii</i> BA 101	~100%	0.03	0.30		6.78	504 h	(Ezeji et al., 2007)
Continuous adsorptive immobilization	<i>C. beijerinckii</i> BA101	33%	2.0	15.8	0.38	7.9 (Total solvents)	600	(Qureshi et al., 2000)
Continuous two stage column with <i>insitu</i> product recovery	<i>C. acetobutylicum</i> B 5313	93.72%	0.2	10.85	0.38	13.6	1152	This study

