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Wood pulp as an immobilization matrix for the continuous production of isopropanol and butanol

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Abstract The study was focused on developing a continuous method to produce an alcohol mixture suitable to be used as a gasoline supplement. The immobilized column reactor with wood pulp fibers was successfully used for the continuous production of butanol and isopropanol using *Clostridium beijerinckii* DSM 6423. A sugar mixture (glucose, mannose, galactose, arabinose and xylose) representing lignocellulose hydrolysate was used as a substrate for the production of solvents. The effect of dilution rate on solvent production was studied during continuous operation. The maximum total solvent concentration of 11.99 g/l was obtained at a dilution rate of 0.16 h^{-1}. The maximum solvent productivity (5.58 g/l h) was obtained at a dilution rate of 1.5 h^{-1}. The maximum solvent yield of 0.45 g/g from sugar mixture was observed at 0.25 h^{-1}. The system will be further used for the solvent production using wood hydrolysate as a substrate.

Keywords Isopropanol · *n*-butanol · Wood pulp · Immobilization · *Clostridium beijerinckii*

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Introduction

The ABE (acetone butanol ethanol) fermentation used to be the second largest industrial scale fermentation process, but it was replaced by petrochemical methods in 1960s. The reasons behind this shift included low ABE productivity (slow fermentation), low solvent yield and substantially high cost of recovery. In the last few decades, interest in bio fuels has been renewed because of the environmental concerns and diminishing petroleum supplies. Butanol is an excellent feedstock chemical in the plastics industry and, more importantly, a superior fuel compared to ethanol. It contains 22 % oxygen making it a beneficiary fuel extender that burns cleaner than ethanol [14]. Isopropanol is said to be one of the four short chain aliphatic alcohols that may become the major feed stocks for the future chemical industry. It can also be used as a fuel additive for the preparation of high-octane gasoline [12, 18]. The separation of isopropanol and butanol as compared to the ABE mixture is also easier because of their properties. Acetone in ABE mixture is not suitable as a fuel due to its corrosiveness to the rubber or plastic parts of engine; therefore, its coproduction with butanol is viewed as undesirable. To increase the butanol yield per unit mass of substrate utilized by reducing acetone production has been an important objective of clostridial metabolic engineering [10, 13].

Recently there have been some successful attempts to engineer clostridia, which can produce isopropanol instead of acetone [2, 10]. Isopropanol is also produced naturally together with butanol and ethanol by several solventogenic clostridia [1, 5, 6, 21]. However, production optimization studies on isopropanol producing clostridia are limited. This has motivated us to develop a continuous process for the production of isopropanol and butanol mixtures using *Clostridium beijerinckii*.

The traditional ABE batch fermentation process experiences problems such as low cell density, low reactor productivity, high down times, nutritional limitations and severe product inhibition, which hinder its commercial development. These problems can be overcome in the immobilized cell reactors. Immobilized cell technology has been extensively investigated and its application has been reported for the production of solvents [8, 11, 16, 17, 22–24, 28]. The immobilization of cells can be done by adsorption or entrapment, but immobilization by passive adhesion to surfaces was found to be more preferred. This technology requires the use of cheap and easily available carriers.

In the present study, various lignocellulosic matrices were screened in batch experiments and wood pulp was selected for the continuous operation. *C. beijerinckii* cells were immobilized on wood pulp fibers and used for continuous production of solvents. The continuous production of isopropanol and butanol using immobilized column technology is not well documented in the literature. Solvent production was studied using sugar mixture instead of a single substrate. The performance of the immobilized cell reactor was investigated for the production of butanol and isopropanol. The effect of various dilution rates on solvent production, yield and substrate utilization was studied.

Materials and methods

Materials

Glucose was purchased from VWR International, Finland. Yeast extract and tryptone were purchased from Lab M Ltd, UK. *p*-amino benzoic acid, $MgSO_4$, $FeCl_3$, $NaMoO_4$ and $CaCl_2$ were obtained from Fluka, Switzerland. The L-cysteine hydrochloride and biotin were purchased from Sigma Aldrich, USA. The K_2HPO_4, sodium sulphate, $ZnSO_4$, $CuSO_4$, were obtained from Merck, Germany. The NaOH, HCl and H_2SO_4 were obtained from J.T. Baker, Holland. All the chemicals were analytical grade. The wood pulp fibers were obtained from Department of Forest Products Technology, Aalto University School of Chemical Technology, Espoo, Finland. The pulp fibers were prepared by using the SO_2–ethanol–water (SEW) fractionation method [20].

Microorganism and inoculum preparation

Clostridium beijerinckii DSM 6423 was obtained from DSMZ, Germany (German Collection of Microorganisms and Cell Cultures). Frozen stock cultures containing 50 % (w/v) glycerol were stored in 2 ml ampoules at −70 °C. The culture was inoculated (2 % v/v) to 100 ml of medium in 125 ml air tight, anaerobic glass bottles and grown for 20 h at 37 °C. This was used to inoculate the batch fermentation medium. One liter of inoculum was centrifuged and biomass was suspended in fresh medium to recirculate in the column reactor for immobilization.

Medium

The medium reported by George et al. [6] was used with slight modification for the inoculum preparation and as a production medium. The inoculum medium contained glucose 30 g/l. Modified production medium contained a sugar mixture of glucose, mannose, arabinose, galactose and xylose instead of a single carbon source. It contained (in g/l) glucose 8.5, mannose 22.0, arabinose 2.3, galactose 4.5 and xylose 10.50, yeast extract 5.0, tryptone 1.0, sodium sulphate 0.18, K_2HPO_4 3.48, *p*-amino benzoic acid 0.01, cysteine-hydrochloride 0.5, biotin 0.01 and 1 ml per liter of mineral solution. The mineral solution contained (in 1 l) $NaMoO_4{\cdot}2H_2O$, 2.4 g, $CoC1_2{\cdot}6H_2O$, 0.24 g; $CaCl_2{\cdot}2H_2O$, 1.5 g; $FeCl_3{\cdot}6H_2O$, 27 g; H_2SO_4, 28 ml; $CuSO_4{\cdot}5H_2O$, 0.25 g; $ZnSO_4{\cdot}7H_20$, 0.29; $MnSO_4{\cdot}H_2O$, 1.7 g; and $MgSO_4$, 12 g. The medium was adjusted to pH 6.8 with HCl before autoclaving. After preparation, the medium was purged with oxygen free nitrogen and autoclaved at 10^5 Pa (121 °C) for 20 min and cooled.

Batch fermentation

Batch fermentations were carried out in a 1L jacketed bioreactor (Braun Biostat Q) with the fermentation volume of 500 ml. The culture medium was purged with nitrogen to maintain the anaerobic conditions and autoclaved at 10^5 Pa (121 °C) for 20 min and cooled. It was inoculated with a 10 % v/v cell suspension which was grown for 20 h at 37 °C as mentioned in the section on microorganism and inoculum preparation. Samples were taken every 12 h from batch fermentation for biomass and product analysis. The pH was maintained at 4.8 ± 0.2 using 3 M NaOH.

Batch fermentation using immobilization matrices

Different lignocellulosic support materials such as wood pulp, loofa sponge, coconut fibers, wood chips and sugarcane bagasse were evaluated. The matrices (except wood pulp) were cut into 3–5 mm particle sizes and washed with water three times and dried in an oven at 70 °C. Processed matrices were added to the production medium at a ratio of 1:4 v/v in 125 ml air tight bottles. Inoculation was done (5 % v/v) with 20 h actively growing seed culture after autoclaving at 121 °C for 20 min and the culture was incubated for 120 h at 37 °C.

Continuous reactor operation

The wood pulp was chosen as an immobilization material based on the previous experiments. The wet wood pulp was rolled inside the plastic mesh and inserted into the glass column. The whole immobilization matrix was sterilized with 70 % ethanol for 24 h and used for immobilization of cells. The immobilization was performed using a concentrated cell suspension, which was recirculated for 24 h. The production medium was continuously fed to the immobilized cell reactor at different dilution rates. After changing the dilution rate, the culture was allowed to stabilize indicated by stable solvent and acid production and substrate consumption. The samples were taken from the top of the column and centrifuged at 15,000 rpm for 5 min and supernatants were used for the substrate and product analysis. The column temperature was maintained at 37 °C by continuously circulating water through the jacket.

Determination of substrates and products

The produced solvents and acids were quantified by using gas chromatography. The gas chromatograph (Hewlett Packard series 6890) equipped with a flame ionization detector was used. Separation took place in a DB-WAXetr capillary column (30 m × 0.32 mm × 1 μm) from Agilent Technologies, Finland. The injector temperature was 200 °C and detector temperature was 250 °C. The injector volume was 10 μl. Glucose, mannose, arabinose, galactose and xylose were determined by high-performance liquid chromatography (Biorad 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 H_2SO_4) was circulated with a flow rate of 0.60 ml min^{-1}. 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.

Calculation of bioprocess parameters

Dilution rate in h^{-1} was calculated as a flow rate divided by the working volume of the column. The overall solvent productivity in g/l h during continuous cultivation of solvent-producing clostridia was expressed as g/l of total solvents multiplied by the dilution rate (h^{-1}). Solvent yield was calculated by dividing the total solvents in g/l by the utilized substrate in g/l. Initial substrate concentration of 45 g/l in the fresh medium was used to calculate the amount of substrate utilized.

Results and discussion

Many researchers [7, 21, 24, 26] have reported that the clostridium can adsorb and grow on lignocellulosic materials without any additional chemicals. The use of sugar mixture and wood pulp as an immobilization material was studied. This information will be beneficial for the wood based bio refinery. Pulp can be sold as such and spent liquor produced during pulping process can be used for the production of solvents. Another advantage could be that the spent pulp can be hydrolyzed for sugar production, thus minimizing waste generation.

The time course of batch fermentation (Fig. 1) showed that the solvent production started at the end of the exponential phase (after 6 h). Batch culture gave the maximum concentration of 1.20 g/l of isopropanol and 2.78 g/l of butanol after 48 h of the fermentation with no further significant increase even after increasing the fermentation time. The culture broth also showed the slight presence of ethanol (<0.1 g/l) and, hence, was not reported. The maximum solvent yield was found to be 0.25 g/g with only 34.9 % substrate conversion (residual sugars 29.3 g/l). The results showed that *C. beijerinckii* successfully utilized all the sugar components in the mixture. After 48 h of cultivation, the residual sugars were as follows (in g/l): glucose 2.3, mannose 15, arabinose 1.6, xylose 6.5 and galactose 3.9 indicating 71, 32, 31, 25 and 5 % sugar consumption, respectively. This confirms that glucose was the most favored carbon source followed by mannose, arabinose and xylose, whereas galactose was found to be the least preferred. The concentration of different sugar components was selected on the basis of results obtained by SO_2–ethanol–water fractionation process of wood biomass [20].

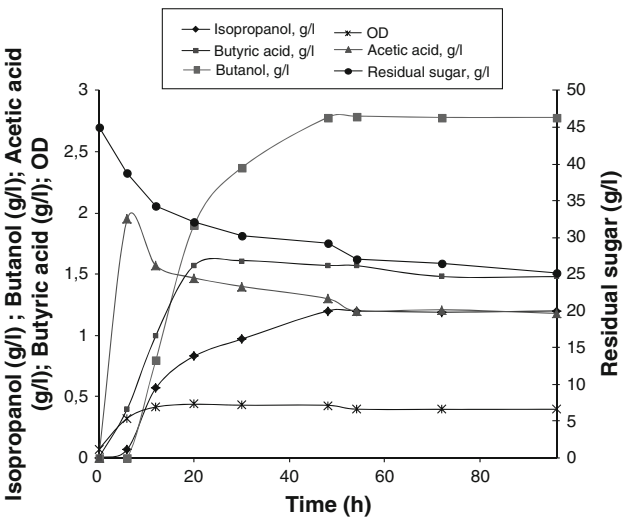

Fig. 1 Course of fermentation in a batch culture of *C. beijerinckii* DSM 6423

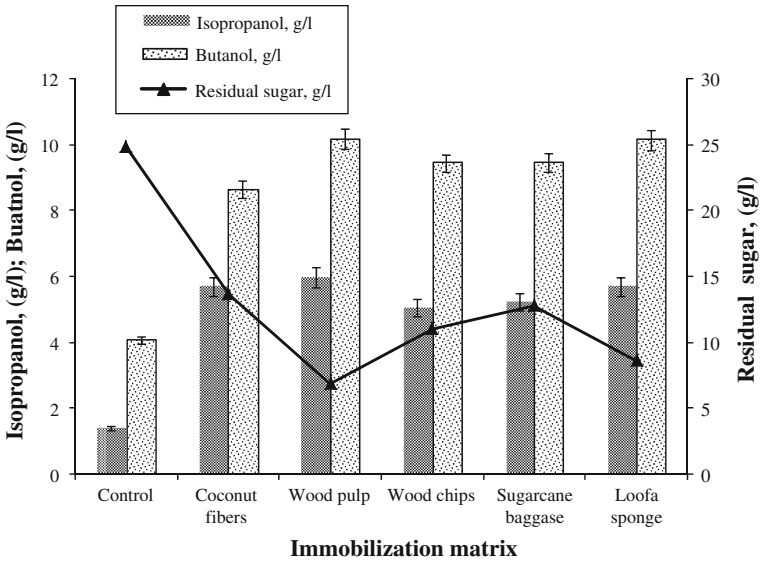

Fig. 2 The effect of addition of different immobilization materials on isopropanol and butanol production and glucose utilization with *C. beijerinckii* DSM 6423. Control is without adding any support matrix

The results from the screening of various immobilization materials in batch experiments are shown in Fig. 2. It was found to be beneficial to add the support material in batch fermentation. All the different materials screened gave the improved production of isopropanol and butanol compared to the control, where no holding material was added. Survase et al. [23] reported previously that the coconut fibers and wood pulp are the most promising support matrices for Clostridia. The maximum ABE concentration of 18.88 and 18.60 g/1 with the wood pulp and coconut fiber, respectively, was reported. Tripathi et al. [24] and Shamsudin et al. [19] reported importance of the addition of support materials and increased production of solvents using this method. All the materials screened fulfill the requirements of an ideal immobilization support matrix including nontoxic nature, easy availability, reusability and high surface area for cell attachment. The adsorption capacity and strength of binding are also important factors that determine the selection of a suitable support material.

Results for isopropanol and butanol production and substrate utilization are shown in Fig. 3. The solvent concentration (average for three consecutive days at each dilution rate after reaching the steady state) varied between 1.88 to 11.99 g/l over a selected dilution rate range from 0.16 to 2.0 h^{-1}. The concentration of total acids (acetic and butyric) ranged from 1.03 to 3.41 g/l over a dilution range of 0.16 to 2.0 h^{-1}. Residual sugars ranged from 18.6 to 39.4 g/l. Maximum solvent production of 11.99 g/l was obtained at a dilution rate of 0.16 h^{-1} with the 18.6 g/l

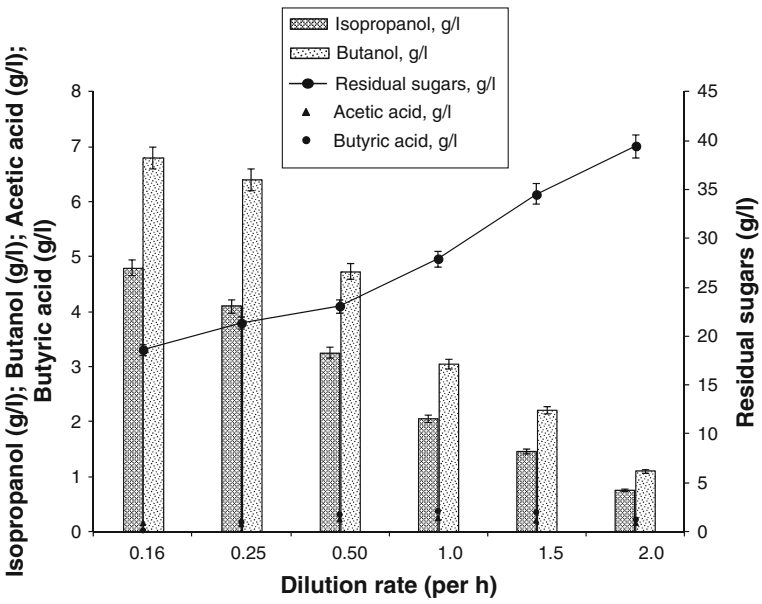

Fig. 3 The effect of different dilution rates on isopropanol, butanol and acids production and sugar utilization during the continuous production of butanol and isopropanol in the immobilized cell reactor containing *C. beijerinckii* DSM 6423

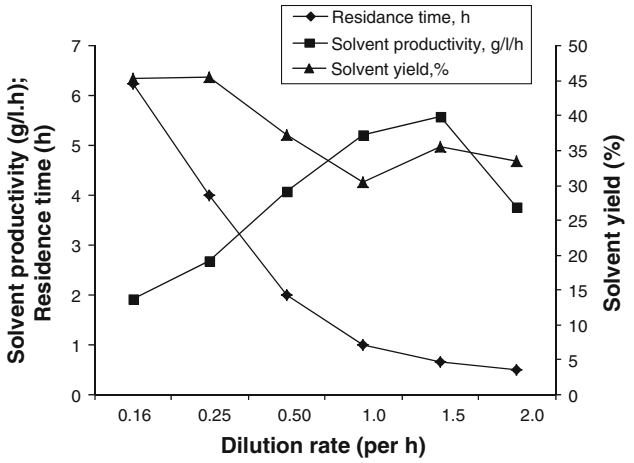

Fig. 4 The effect of different dilution rates on solvent productivity, solvent yield and average residence time during the continuous production of butanol and isopropanol in the immobilized cell reactor containing *C. beijerinckii* DSM 6423

total residual sugars. The overall solvent productivity, solvent yield and average residence time of production medium are depicted in Fig. 4. Increasing the dilution rate from 0.16 to 1.5 h^{-1} resulted in an increased solvent productivity from 1.91 to 5.58 g/l. h while a further increase from 1.5 to 2.0 h^{-1} resulted in decreased productivity to 3.76 g/l h. The highest solvent yield from sugar mixture (0.45 g/g or 45 %) was obtained at a dilution rate of 0.25 h^{-1}. The solvent yield was found to be better than reported (0.3 g/g) for isopropanol producing *Clostridia* [9]. Formanek et al. [4] reported solvent yield of 0.48 g/g with *C. beijerinckii* BA101 using 6 % glucose as a substrate. While building the column reactor, pulp obtained from SEW fractionation method was used. The pulp was not processed further before using it for column construction. The adsorbed sugars into the pulp could be one reason for improved solvent yield. Other possible reason could be the utilization of pulp fibers by *C. beijerinckii,* but there are no reports on utilization of cellulose by *C. beijerinckii.* Although there are reports on the use of cellulose as a substrate by different *Clostridium* strains [25, 27].

The average residence time of medium in the column reactor ranged from 0.5 to 6.5 h over a range of dilution rates. Krouwel et al. [9] studied continuous production of *n*-butanol and isopropanol by immobilized growing *C. butylicum* cells. They immobilized cells in the Ca-alginate beads and packed them into the conical column and studied the solvent production. They reported the substrate conversion of less than 40 % and yield of about 30 %. In our previous study [21] we used wood pulp as a cell holding material in a single stage continuous bioreactor, where pulp prevented the cell loss and allowed the system to operate at higher dilution rate. High surface area and the lignocellulosic nature might be the advantage of using wood pulp as an immobilization or cell holding material. The disadvantage of this system was cell washout above the dilution rate of 0.8 h^{-1} which resulted in decreased productivity and substrate utilization.

Table 1 reports the utilization of different sugars from the mixture at varied dilution rates. It was confirmed from the continuous production experiments that glucose is the most favored substrate. Among others mannose, arabinose and xylose were also consumed, but galactose was found to be the least preferred. Above dilution rate 0.5 h^{-1} galactose consumption was insignificant. Substrate consumption was minimal with increasing dilution rate. It can be observed from the study that sufficient residence time should be given to sugars to get used to produce solvents. Ezeji and Blaschek [3] reported the consumption of sugar mixtures by clostridia with glucose being the most preferred one. The importance of excess availability of fermentable sugars in the broth was also reported for both the onset and the maintenance of solvent production. Otherwise the fermentation becomes acidogenic leading to premature termination of the fermentation process.

Table 1 The utilization of different sugars at different dilution rates during continuous production of butanol and isopropanol in the immobilized cell reactor containing *C. beijerinckii* DSM 6423

Dilution rate (h^{-1})	Remaining sugars (g/l)						Substrate consumption (%)
	Glucose	Mannose	Galactose	Arabinose	Xylose	Total	
Initial[a]	7.9	22.0	4.1	2.3	8.7	45.0	0.0
0.16	0.2	7.3	3.6	0.7	6.8	18.6	58.7
0.25	0.3	9.5	3.8	0.9	6.9	21.4	52.6
0.5	0.7	10.2	4.0	1.0	7.1	23.1	48.8
1.0	1.6	13.6	4.0	1.1	7.6	27.9	38.1
1.5	3.8	17.8	4.0	1.1	7.8	34.5	23.3
2.0	5.3	19.6	4.1	1.8	8.3	39.4	12.4

[a] Initial sugar concentrations reported are after autoclaving the production medium. All values are an average of 3 day readings obtained after culture has reached the steady state

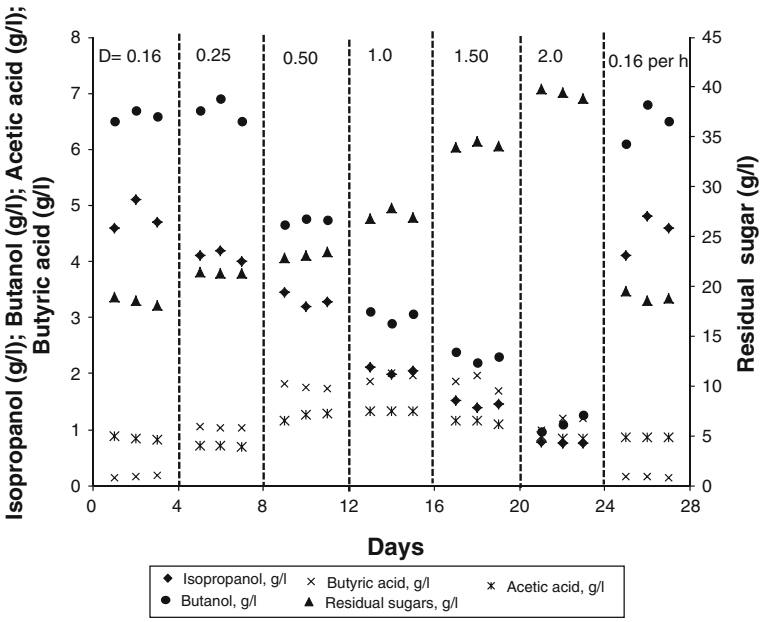

Fig. 5 Time course graph showing the production of isopropanol, butanol and acids and sugar utilization in the immobilized cell reactor containing *C. beijerinckii* DSM 6423

Table 2 Comparison of continuous reactor performance with the different cell immobilization technique

Immobilized cell system	Strain used	Total solvents (g/l) (solvents produced[a])	Productivity (g/l.h)	Yield (g/g)	Operation (days)	Reference
Brick	*C. beijerinckii* BA101	7.9 (ABE)	15.80	0.38	25	[17]
Bonechar	*C. acetobutylicum*, P262	6.5 (ABE)	6.5	0.38	25	[15]
Fibrous bed	*C. acetobutylicum* ATCC 55025	8.5 (ABE)	7.6	0.53	22	[7]
Corn stalk	*C. beijerinckii* ATCC 55025	5.1 (ABE)	5.06	0.32	20	[28]
Ca-alginate beads	*C. butylicum LMD* 27.6.	<5.0 butanol and isopropanol)	4 × batch fermentation	Approx 0.3	9[b]	[9]
Wood pulp fibers	*C. beijerinckii* DSM 6423	5.22 (butanol and isopropanol)	5.22	0.30	27	This work

[a] *ABE* acetone, butanol and ethanol; [b] retention times

The continuous reactor was operated over a period of over 25 days. Figure 5 depicts the time course showing the production of isopropanol, butanol and acids and the sugar utilization in the immobilized cell reactor. The dilution rate was then adjusted back to 0.16 h^{-1} to confirm that the reactor was still working. We could get the maximum solvent concentration of 11.87 g/l suggesting that the reactor can be used continuously for a long time. There was continuous cell growth in the column. The detached cells were observed in the product stream, but the cell concentration never exceeded 0.5 g/l. The decrease in the productivity at a dilution rate of 2.0 h^{-1} was due to insufficient contact time between cells and the feed medium. This was evidenced by the increased production of acids at this dilution rate.

Continuous cultures with immobilized biomass or biomass retention have been reported to give high solvent productivities due to high dilution rates [11, 17, 23, 28]. Similar results were found in the present study. Welsh et al. [26] reported that immobilized cell systems are able to maintain the high cell concentrations, generally have improved reaction rates, and are stable at high dilution rates with minimum cell washout. Other advantages are that the reactor configuration can be relatively simple and materials used for construction can often be reused, Krouwel et al. [8]. The performance of different immobilization systems for the continuous production of solvents is given in Table 2. From the previous reports it can be said that the surface area provided by the immobilization materials plays an important role to make the continuous production effective.

Conclusions

Wood pulp fibers could be successfully used as an immobilization material for the continuous isopropanol and butanol production using sugar mixture as a substrate. A

high solvent productivity of 5.58 g/l h was obtained with the solvent yield of 0.35 g/g. The solvent production can be improved with the better solvent producing clostridia. The bioreactor was operated for nearly 25 days in the continuous flow mode. The efforts to prolong the operation time are in progress to make the process industrially feasible. The studies using wood hydrolysate will be carried out in the future using this system.

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