0273-2289/04/114/0713-0721/$25.00

High-Productivity Continuous Biofilm Reactor for Butanol Production

Effect of Acetate, Butyrate, and Corn Steep Liquor on Bioreactor Performance

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Abstract

Corn steep liquor (CSL), a byproduct of the corn wet-milling process, was used in an immobilized cell continuous biofilm reactor to replace the expensive P2 medium ingredients. The use of CSL resulted in the production of 6.29 g/L of total acetone-butanol-ethanol (ABE) as compared with 6.86 g/L in a control experiment. These studies were performed at a dilution rate of 0.32 h^{-1}. The productivities in the control and CSL experiment were 2.19 and 2.01 g/(L·h), respectively. Although the use of CSL resulted in a 10% decrease in productivity, it is viewed that its application would be economical compared to P2 medium. Hence, CSL may be used to replace the P2 medium. It was also demonstrated that inclusion of butyrate into the feed was beneficial to the butanol fermentation. A control experiment produced 4.77 g/L of total ABE, and the experiment with supplemented sodium butyrate produced 5.70 g/L of total ABE. The butanol concentration increased from 3.14 to 4.04 g/L. Inclusion of acetate in the feed medium of the immobilized cell biofilm reactor was not found to be beneficial for the ABE fermentation, as reported for the batch ABE fermentation.

Index Entries: Immobilized cell biofilm reactor; butanol; corn steep liquor; sodium butyrate; *Clostridium beijerinckii* BA101; sodium acetate.

†Names are necessary to report factually on available data. However, the USDA neither guarantees nor warrants the standard of the product; and the use of the names by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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Introduction

Butanol, an industrially important chemical, can be produced by fermentation of carbohydrates using various solventogenic clostridia. In fact, this fermentation was commercially viable until after World War II when petrochemically produced butanol became available at competitive prices (*1*). For several reasons, including fluctuating oil prices and the depletion of oil reserves, intensive research efforts on this fermentation have been made. Recent technological developments including the development of superior cultures, productive reactors, and efficient downstream processing have once again made this fermentation attractive. Although these developments are encouraging, certain problems, such as the use of economical substrates, need to be resolved before this fermentation will be able to become commercially viable.

The use of continuous immobilized cell biofilm reactors eliminates downtime and hence results in superior reactor productivity (*2,3*). Adsorbed cell continuous biofilm reactors have been shown to favorably affect process economics (*4*). Application of these reactors reduces capital and operational cost, thus making the process simpler. Within these reactors, cells are immobilized by adsorption, which is a simpler technique than other techniques such as entrapment and covalent bonding (*5*). Adsorption is a simple technique and can be performed inside the reactors without the use of chemicals, whereas entrapment and covalent bonding are complicated techniques and require chemicals for bond formation. In anaerobic systems, such as butanol production, adsorption can be performed anaerobically within the reactor. An additional advantage of adsorption is that cells form uniform biofilm layers around the support, which lessens diffusion resistance compared to entrapped and covalently bonded cells. Hence, these reactors are called biofilm reactors. Because of reduction in diffusion resistance, the reaction rate is enhanced. For this reason, adsorption was chosen as the technique to be employed for *Clostridium beijerinckii* BA101 cell immobilization to produce butanol. In addition to being simple, it has the potential to be used in large-scale reactors. In the present study, clay brick was chosen as the cell adsorption support. It is available at a low cost and is easy to dispose of after use.

The previous cited studies were performed using P2 medium, which is composed of expensive chemicals such as biotin, thiamin, and yeast extract. The use of these chemicals at the commercial level would not be commercially viable. Hence, it is suggested that the use of cheaper and simpler nutrient sources be investigated. The use of corn steep liquor (CSL) has been reported in pilot plant trials for butanol batch production employing *C. beijerinckii* BA101 (*6*). CSL is a byproduct of the corn wet-milling process and contains nutrients leached out of corn during the soaking process. The reader is advised that there are few studies on the use of CSL in continuous immobilized cell biofilm reactors. Similarly, the use of exogenous sodium butyrate and sodium acetate has not been reported in continuous immobilized cell biofilm reactors. Hence, these studies are

considered novel. The successful application of CSL, sodium butyrate, and sodium acetate is expected to benefit the economics of butanol production by fermentation.

One objective of the present study was to examine the effect of CSL on the performance of high-productivity biofilm reactors. Another objective was to study the effect of supplementing acetate and butyrate into the feed of the biofilm reactor. Supplementation of acetate to the feed medium of batch reactors (not continuous biofilm reactors) has been shown to be beneficial to this fermentation. It is also anticipated that butyrate added to the feed would be converted to butanol.

Materials and Methods

Organism and Culture Maintenance

For fermentation studies, the microorganism used was the *C. beijerinckii* BA101 hyperbutanol-producing strain *(7)*. Laboratory stocks of *C. beijerinckii* BA101 were routinely maintained as spore suspensions in sterile double-distilled water at 4°C. *C. beijerinckii* BA101 spores (200 µL) were heat shocked in 50 mL of cooked meat medium (CMM) for 10 min at 80°C followed by cooling in ice-cold water. Pyrex bottles (100-mL volume) containing heat-shocked spores were incubated anaerobically for 15 to 16 h at 36 ± 1°C before inoculating the reactor.

Fermentation Media

The feed medium contained glucose (60 g/L), yeast extract (Difco, Detroit, MI) (1 g/L), and P2 medium ingredients (buffer, minerals, and vitamins) *(8)* unless stated otherwise. Glucose and yeast extract solutions were sterilized separately at 121°C for 15 min followed by cooling to room temperature by sweeping oxygen-free N_2 gas across the surface of the medium. Stock solutions of buffer, mineral, and vitamins were added aseptically to the cooled glucose-yeast extract solution. The stock solutions were filter sterilized through a 0.2-µm filter. The medium was kept anaerobic by continuously sweeping oxygen-free N_2 across the surface. When needed, sodium butyrate or sodium acetate solutions were added to the P2 feed medium. The pH of the feed medium was adjusted to 6.8 prior to autoclaving.

Thirty-two grams of CSL (500 g/L of solids slurry) was added to each liter of CSL medium. The composition of CSL has been given elsewhere *(9)*. The CSL was dissolved in approx 300 mL of water and boiled for 30 min. After cooling to room temperature, the mixture was centrifuged to remove solids. To the supernatant, 60 g of glucose was added and the volume adjusted to 950 mL with water. The pH of the feed solution was adjusted to 6.8 prior to autoclaving at 121°C for 15 min. The autoclaved medium or solutions were cooled anaerobically by sweeping oxygen-free N_2 gas across the surface. One gram of cysteine hydrochloride was dissolved in 25 mL of water and filter sterilized (0.2 µm) before aseptically adding to the cooled

CSL medium. Twenty-five milliliters of $FeSO_4 \cdot 7H_2O$ (0.012 g) solution was made in water followed by filter sterilization (0.2 μm) and adding to the CSL medium. $FeSO_4 \cdot 7H_2O$ is an essential mineral for solventogenesis and was included in both P2 and CSL media. The CSL medium was fed to the reactor.

Bioreactor and Cell Immobilization

The reactor was composed of a 312-mL total volume jacketed polyacrylic (192 × 46 mm) vessel. The reactor was sterilized using 30% (v/v) ethanol solution for 48–72 h, after which it was drained and washed thoroughly with sterilized deionized water. Brick pieces (4 to 5 mm, total weight of 220 g) were washed with deionized water several times followed by sterilization in an oven at 250°C for 2 h. The particles were then cooled in an anaerobic chamber to room temperature. The void volume inside the reactor was 186 mL. The reactor was packed aseptically with the sterilized brick particles. After the reactor was packed, oxygen-free N_2 was passed through the column overnight. This was done to ensure that anaerobic conditions were attained inside the column and inside the particles.

Forty milliliters of actively growing *C. beijerinckii* cells from a CMM bottle were inoculated into the reactor, and the reactor was filled with fresh P2 medium. The composition of P2 medium has been published elsewhere *(8)*. Cell growth was allowed inside the reactor for 4 h, after which the P2 medium was continuously fed (using a peristaltic pump [Cole-Parmer, Vernon Hills, IL] and silicone tubing) at a flow rate of 92 mL/h (dilution rate of 0.29 h^{-1}). Water (35°C) was circulated through the jacket of the column to control temperature using a circulating water bath (Polystat; Cole-Parmer). Samples were taken after 3 d. The dilution rate was altered whenever a steady state was reached in terms of average solvent production and glucose utilization. Samples were collected over a period of five to seven steady-state residence times, with a sampling frequency of one residence time. Samples were centrifuged at 16,000*g* for 1 to 2 min. The supernatant was stored at −18°C in preparation for acetone, butanol, ethanol, and glucose analysis. Comparisons of the media were made in the same reactor (not in parallel reactors). The pH of the reactor effluents was not measured. The reader is advised that the pH inside the bioreactor was not controlled. Solventogenic clostridia regulate pH at approx 5.0. A schematic diagram of the reactor is shown in Fig. 1.

Analyses

Cell concentration in the reactor effluent was estimated by optical density (OD) and cell dry weight method using a predetermined correlation between OD at 540 nm and cell dry weight. Acetone-butanol-ethanol (ABE) and acids (acetic and butyric) were measured using a 6890 Hewlett-Packard gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector and 6 ft × 2 mm glass column (10% CW-20M, 0.01% H_3PO_4, support 80/100 Chromosorb WAW). Productivity was calcu-

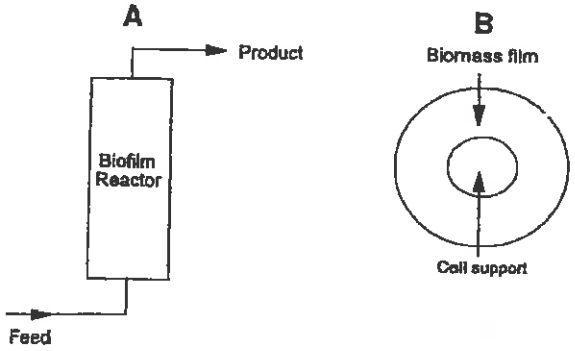

Fig. 1. Schematic diagram of butanol production in an immobilized cell biofilm reactor using *C. beijerinckii* BA101: (A) bioreactor; (B) biofilm particle of *C. beijerinckii* BA101.

lated as the average ABE concentration multiplied by dilution rate (h^{-1}). Dilution rate was defined as feed flow rate per reactor volume and is based on the total volume of the reactor. ABE yield was defined as grams of ABE produced per gram of glucose utilized.

Glucose concentration was determined using a hexokinase- and glucose-6-phosphate dehydrogenase-- (Sigma, St. Louis, MO) coupled enzymatic assay. The fermentation broth was centrifuged (microfuge centrifuge) at 16,000*g* for 3 min at 4°C. A portion of the supernatant (10 µL) was mixed with glucose (HK) 20 reagent (1.0 mL) and incubated at room temperature for 5 min. Standard solutions of anhydrous D-glucose containing 1–5 g of glucose/L of distilled water were prepared. Ten microliters of each of the standard solutions was mixed with glucose (HK) 20 reagent (1.0 mL) and incubated at room temperature for 5 min. A blank (deionized water) (10 µL) was incubated with the reagent and used for zero adjustment of the spectrophotometer. After 5 min, the absorbance was measured at 340 nm using a Beckman DU 640 spectrophotometer, and the glucose content in the sample was computed by least squares linear regression using a standard curve.

Results and Discussion

Our previous biofilm reactor studies suggested that basal medium (1 g/L of yeast extract + 60 g/L of glucose) was a poor nutrient medium and did not promote good solventogenesis (*5*). The reactor fed with this medium produced 3.75 g/L of total solvents compared with 11.90 g/L using P2 medium. This indicated that P2 or CSL medium may be required in this reactor design in order to produce solvents. As a result of those studies, the following studies were performed using P2 or CSL medium.

Table 1
Effect of CSL, Butyrate (NaBu), and Acetate (NaAc) on Butanol Production in Immobilized Cell Biofilm Reactor of *C. beijerinckii* BA101

Fermentation parameters and products	Dilution rate (0.32 h^{-1})					
	CSL		Butyrate		Acetate	
	Control	CSL	Control	NaBu[a]	Control	NaAc[b]
Acetone (g/L)	2.61	2.00	1.51	1.55	3.20	2.44
Butanol (g/L)	4.12	4.16	3.14	4.04	5.76	3.49
Ethanol (g/L)	0.13	0.13	0.12	0.11	0.16	0.12
Acetic acid (g/L)	1.83	1.84	0.42	2.70	0.40	2.45
Butyric acid (g/L)	0.53	0.45	1.14	0.85	0.73	1.83
Total ABE (g/L)	6.86	6.29	4.77	5.70	9.12	6.05
Total acids (g/L)	2.36	2.29	1.56	3.55	1.14	4.28
Glucose in feed (g/L)	65.50	67.50	59.10	59.70	64.40	64.40
Glucose utilization (%)	39.10	30.50	29.40	35.20	40.00	38.00
ABE Yield (g/g)	0.27	0.30	0.50	0.27	0.35	0.22
Productivity (g/(L·h))	2.19	2.01	1.53	1.82	2.92	1.94

[a] 2.5 g/L of butyrate added.
[b] 6.56 g/L (80 m*M*) of acetate added.

Initially the reactor was fed at 92 mL/h (dilution rate of 0.29 h^{-1}) with P2 medium until there were visible signs of cell mass accumulation. It took about 5 d to accumulate cell mass and for the reactor to be productive. The flow rate was then increased to 100 mL/h, and the reactor was allowed to achieve a new steady state. At this stage, a control experiment was run and the reactor was fed with P2 medium. At this dilution rate (0.32 h^{-1}), the reactor produced 6.86 g/L of total ABE and 2.36 g/L of total acids (Table 1). This resulted in an ABE productivity of 2.19 g/(L·h) and a yield of 0.27. This productivity is manyfold higher than the productivity achieved in a batch reactor (0.38 g/[L·h]) *(10)*. Glucose utilization was 39.1% of that available in the feed (65.5 g/L). In these reactors, high productivity is achieved but at the expense of a low ABE concentration in the effluent as well as low sugar utilization. To improve sugar utilization, the reactor effluent should be recycled back to the reactor after ABE removal *(11)*.

To compare the performance of the reactor and evaluate the effect of CSL incorporation into the feed, the reactor was fed with CSL medium. Fermentation conditions and the dilution rate were kept constant as in Table 1 for the duration of this experiment. The reactor produced 6.29 g/L of total ABE, of which acetone, butanol, and ethanol were 2.00, 4.16, and 0.13 g/L, respectively (Table 1). This resulted in a productivity of 2.01 g/(L·h) and a sugar utilization of 30.5% of that available in the feed (67.5 g/L). Compared to the control, the productivity was reduced by 10%. However, it is anticipated that it would be economical to use CSL compared with the P2 medium. This demonstrated that P2 medium can be replaced by economically available CSL. It is suggested, however, that the CSL

medium be optimized for further enhancement of ABE production in the immobilized cell reactor. It is also suggested that CSL be used to initiate the reactor and study the effect of CSL on cell growth and cell immobilization.

Next, an experiment was run in which 2.5 g/L of sodium butyrate was added to P2 medium to investigate whether it could be converted to butanol. A control experiment was run containing P2 medium. A separate control experiment was run before each experiment. This is essential because biomass accumulation in the reactor changes with time, thus affecting performance of the reactor *(5)*. The reactor produced 4.77 g/L of total ABE, of which acetone, butanol, and ethanol were 1.51, 3.14, and 0.12 g/L, respectively (Table 1). It resulted in a total ABE productivity of 1.53 g/(L·h) and a glucose utilization of 29.4% of that available in the feed of 59.1 g/L. The acid concentration in the effluent was 1.56 g/L. Following this, P2 medium was supplemented with sodium butyrate and the experiment was conducted at the same dilution rate. The reactor produced 1.55 g/L of acetone, 4.04 g/L of butanol, and 0.11 g/L of ethanol, for a total ABE concentration of 5.70 g/L, compared with 4.77 g/L in the control experiment. The productivity was 1.82 g/(L·h), compared with 1.53 g/(L·h) for the control experiment. These experiments suggested that butyrate was used by the culture to produce additional butanol. Note that 0.9 g/L of butanol was produced from 1.65 g/L of butyrate (2.5 g/L in feed, 0.85 g/L in effluent). The yield calculations do not include the amount of butyrate that was utilized by the culture.

In a previous study in which two reactors connected in series were used to produce butanol by *Clostridium acetobutylicum* P262, acids produced in the first reactor were converted to solvents (ABE) in the second reactor *(12)*. Ramey et al. *(13)*, and Ramey *(14)* used two immobilized cell series reactors to produce butanol from glucose. The first reactor contained immobilized cells of *Clostridium tyrobutyricum* to convert glucose to butyric acid and the second reactor contained immobilized cells of C. *acetobutylicum* ATCC 55025 to convert butyric acid (produced in the first reactor) to butanol. The product of the first reactor was fed to the second reactor without cell separation. It has been reported that the use of continuous two-stage reactors results in a significant increase in butanol yield. Furthermore, it has been demonstrated that application of such reactors eliminates production of all the ancillary byproducts (acetone, ethanol, and acetic acid) associated with butanol fermentation. Our investigations on the use of butyrate were performed with an ultimate objective of converting CO_2 to butyric acid and then to butanol. If successful, this would result in converting 53% of carbon that is lost to CO_2 in butanol fermentation. At this stage, we are attempting to isolate a culture that could convert CO_2 to butyric acid.

It has been reported that incorporation of 4.92–6.56 g/L (60–80 mM) of sodium acetate in the feed medium enhances levels of ABE production in a batch reactor from approx 25 to approx 33 g/L *(15)*. Additionally, it is also reported that levels of a number of key metabolic enzymes are elevated by the inclusion of acetate. Although the objective of the present investigation

was not to study the levels of these enzymes, we were interested in determining whether the inclusion of acetate in the feed medium of an immobilized call biofilm reactor would enhance the overall ABE production levels, thus improving productivity. A control experiment was therefore run with P2 medium. The reactor produced 9.12 g/L of total ABE, of which acetone, butanol, and ethanol were 3.20, 5.76, and 0.16 g/L, respectively. This resulted in a total productivity of 2.92 g/(L·h) at a dilution rate of 0.32 h^{-1}. The glucose utilization was 40% of that in the feed (64.4 g/L) (Table 1). When acetate was included in the feed medium, levels of these solvents decreased. Compared with 9.12 g/L of total ABE production in the control experiment, 6.05 g/L was produced, thus decreasing productivity to 1.94 g/(L·h) (Table 1). Total acid concentration was high, at 4.28 g/L. Interestingly, glucose utilization was 38% of that in feed of 64.4 g/L, similar to the control experiment. Although it has been reported that acetate is beneficial to butanol production in a batch reactor *(15)*, it does not improve the performance of the continuous biofilm reactor. Acetate may have hindered with the self pH regulation near pH 5.0. It may also have inhibited the fermentation owing to an increase in protonated acetate. Cell concentration in the effluent of the reactor varied from 0.6 to 1.5 g/L. We are aware of the significant variations in the results of control experiments. The reasons for these variations have been discussed elsewhere *(5)*. It is suggested that the different controls should not be compared because there have been varying concentrations of biomass in the controls. For these reasons, separate controls were run for all the treatments.

Conclusion

The use of CSL resulted in the production of 6.29 g/L of total ABE, compared with 6.86 g/L achieved in the control experiment. These studies were performed at a dilution rate of 0.32 h^{-1}. The productivities in the control and CSL experiments were 2.19 and 2.01 g/(L·h), respectively. Although the use of CSL resulted in a 10% decrease in ABE productivity, it is viewed that application of CSL would be economical for butanol production by fermentation. It was also demonstrated that inclusion of butyrate in the feed was beneficial to the butanol fermentation. A control experiment produced 4.77 g/L of total ABE, and the experiment supplemented with butyrate produced 5.70 g/L of total ABE. The butanol concentration increased from 3.14 to 4.04 g/L. Inclusion of acetate in the feed medium of the immobilized cell biofilm reactor was not beneficial to the ABE fermentation, as reported previously for the batch ABE fermentation.

Acknowledgments

This work was supported by grants from the Illinois Council on Food and Agricultural Research (IDA CF 02E-073-1 and IFA CF 03E116-1 FSHN) and Illinois Corn Marketing Board (ICMB 00-0126-01 BLASCHEK). CSL was kindly provided by A. E. Staley Manufacturing (Decatur, IL).

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