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SHORT COMMUNICATION

Continuous acetone–butanol–ethanol production by corn stalk immobilized cells

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Abstract Corn stalk was used as a support to immobilize *Clostridia beijerinckii* ATCC 55025 in the fermentation process of acetone, butanol, and ethanol production. The effect of the dilution rate on solvent production was examined in a steady-state 20-day continuous flow operation. The maximum total solvent concentration of 8.99 g l^{-1} was obtained at a dilution rate of 0.2 h^{-1}. Increasing the dilution rate between 0.2 and 1.0 h^{-1} resulted in an increased solvent productivity, and the highest solvent productivity was obtained at 5.06 g l^{-1} h^{-1} with a dilution rate of 1 h^{-1}. The maximum solvent yield from glucose of 0.32 g g^{-1} was observed at 0.25 h^{-1}. The cell adsorption and morphology change during the growth on corn stalk support were examined by the SEM.

Introduction

The production of acetone, butanol, and ethanol (ABE) by fermentation is a process that was used by industry for many decades and used to be the second largest industrial scale fermentation operation. This fermentation process for solvents production was, however, replaced by the petrochemical route in the early 1960s. Due to increasing concern about diminishing petroleum supplies and the impact of petroleum fuel emissions, interest in obtaining solvents from renewable and sustainable resources has recently been renewed with great optimism worldwide.

There are several reasons for the lesser competitiveness of the ABE process compared to the petrochemical route for solvent production, including low ABE productivity, low solvent yield, and the high cost of product recovery. The productivity of a traditional ABE fermentation batch ranges from 0.1 to 0.3 g l^{-1} h^{-1} and requires large fermentors as well as long periods of operation time. A continuous culture system with immobilized cells is a promising technique to improve solvent productivity. There are many techniques for cell immobilization, but two of them are used most often, adsorption and entrapment. Examples of entrapment include using carrageenan [1] and chitosan [2], calcium alginate [3–5], and polyvinyl alcohol [6] as matrix materials, while that of adsorption includes bonechar [7, 8] and brick [9] as absorbent and support materials. One of the most extensively used classes of natural support is lignocellulosic materials. Some lignocellulosic supports being investigated include sawdust, wood chips/shavings, rice husks, cotton towels, and straw [10–14]. The corn stalk is a readily available and inexpensive material with many advantages as a support material for cell immobilization. These advantages include being highly porous as well as having a good water retention capacity. One of the most attractive properties of using cellulosic materials as an immobilization support is that the spent materials can be sent back to the hydrolysis process for sugar production, thus minimizing waste generation. This paper describes the feasibility of using corn stalk as a support and delineates a novel and simple technique for quick cell loading while providing a strong support for immobilized cells.

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Materials and methods

Microorganism and mediums

The asporogenic strain *Clostridia beijerinckii* ATCC 55025 was purchased from America Type Culture Collection. The freeze-dried cultures were rejuvenated according to ATCC's instructions (www.atcc.org). The reinforced clostridial media (RCM, Oxoid CM 149) was used as growing media; 400 ml RCM was placed in a 1,000-ml flask. The flasks with media were sterilized at 121°C for 15 min and were then cooled to room temperature. Followed by overnight retention in an anaerobic chamber in an anaerobic environment (80% N_2, 10% CO_2, and 10% H_2), the media was inoculated with 20 ml rejuvenated cells and incubated for 12 h at 37 ± 1°C with a shaking speed of 90 rpm. The solventogenic media contained 60 g l^{-1} glucose, 1 g l^{-1} yeast extract (Difco Laboratories), and P2 medium ingredients [15]. The solution containing glucose and yeast extract was sterilized at 121°C for 15 min, followed by cooling to room temperature by sweeping oxygen-free N_2 across the surface of the medium. P2 stocks were added to the cooled solution aseptically by filtration sterilization. Then 0.5 g l^{-1} cysteine hydrochloride (Sigma) and 0.0001% (w/v) resazurine (Kodak) were subsequently added to the final media to reduce the redox potential.

Cell immobilization and reaction system setup

The *C. beijerinckii* cells were immobilized by the method of adsorption onto corn stalk. The process for immobilization and the operation of the immobilized cell reactor are shown in a sketch diagram Fig. 1. After removing the husky and the outside fiber, the corn stalk was chopped into small pieces (5–8 mm size) with a knife. The corn stalk was then soaked with 1% (v/v) hydrochloric acid, and the slurry was placed in a 100°C water bath for 30 min. The treated stalk was washed by distilled water twice and dried at 80°C overnight. A 2.0-g harvested stalk was autoclaved at 121°C for 15 min and then packed at room temperature into a 2-cm i.d, 20-cm long plexiglass column that was sterilized at 121°C for 15 min. The packed column was then flushed with anaerobic gas (80% N_2, 15% CO_2, 5% H_2) to remove the residual moisture and O_2 inside the reactor. The reactor was then placed in an anaerobic chamber in which the temperature was controlled at 37 ± 1°C. The slurry of RCM with actively growing *C. beijerinckii* was pumped into the reactor by a peristaltic pump through silicone tubing at a flow rate of 1 ml h^{-1}. The cell and media mixture was then circulated for 24 h for cell adsorption and growth. The storage (buffer) tank was then switched to the solventogenic media tank. The solventogenic media was fed continuously to the immobilized cell reactor at different dilution rates. Samples taken from the top of column were centrifuged at 12,000 rpm for 2 min, and the supernatants were used for the ABE, glucose, and acid content analysis. The utilized glucose was calculated by subtracting the feed concentration by the effluent concentration of glucose.

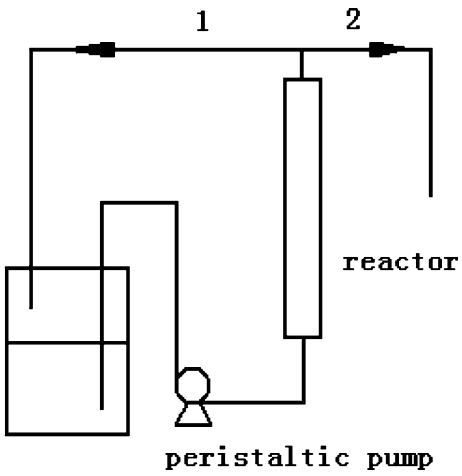

Fig. 1 A diagram of the immobilized cell reactor; when the reactor was circulated with growing media for cell immobilization, the effluent went through pipe 1 back to the media storage tank. During solvent production operation, the storage tank was switched to a tank filled with solventogenic media, and the effluent was discharged through pipe 2

Analytical methods

Glucose and organic acids were determined by a high-pressure liquid chromatograph (HPLC, Agilent model 1050, Agilent, CA) using a 7.8 × 300-mm stainless steel column packed with Aminex HPX-87H ion exclusion packing purchased from Bio-Rad Laboratory (Bio-Rad, USA). Solvents were determined by a gas chromatograph (GC, Varian model 3700, USA) equipped with a Flame Ionization Detector (FID) and Auto Linear Temperature Programmer. The carrier gas was nitrogen. The column was packed with 1-1814 80/120 Carbopack B/6.6% Carbowax 20 M (Supelco, USA).

Scanning electron microscope

For scanning electron microscopy, the samples were fixed with 3.5% (w/v) glutaraldehyde at 4°C for 15 h and washed with distilled water twice followed by a progressive dehydration with 20–100% ethanol at 20% increment, treated with hexamethyldisilazene (HMDS), and sputter-coated with gold-palladium. The samples were scanned and photographed with a scanning electron microscope (KYKY Technology Development Ltd., China) at 15 kV.

Results and discussion

The performance of the immobilized cell reactor was investigated at different dilution rates. At low dilution rates below, for example, 0.5 h^{-1}, the reactor suffered from a severe gas-hold problem around the immobilization support because of CO_2 and H_2 generated by the anaerobic microbe during fermentation. Figure 4 shows SEM of corn stalk with the adsorbed cells at the beginning and the end of the experiment.

Many researchers [12, 13] found that clostridium can automatically stick to the lignocellulosic material and grow on the support without any additional chemicals. The porosity of corn stalk makes the cells not only grow on the surface of the support, but also in the inner side of the supports, resulting in a much higher cell density or cell loading. The corn stalk was treated with diluted HCl aimed to activate the surface by removing the wax and destroy the hemicellulose. The treatment also made the support more porous and increased surface area. Figure 4a shows the adherence of the cells at the end of the cell adsorption operation.

Results for solvents, acids, and utilized glucose concentrations are shown in Fig. 2. The highest glucose utilization of 30.5 g l^{-1} was found at a dilution rate of 0.2 h^{-1}, corresponding to the maximum total solvent concentration of 8.99 g l^{-1}; the concentration of ABE was 2.36, 5.36, and 1.26 g l^{-1}, respectively. Average total solvent productivity and yield are shown in Fig. 3. The total solvent productivity increased from 1.76 to 5.06 g l^{-1} h^{-1} when the dilution rate increased from 0.2 to 1 h^{-1} and decreased to 4.14 at the dilution rate of 2.0 h^{-1}. The highest solvent yield from glucose of 0.32 g g^{-1} was obtained at the dilution rate of 0.25, and the yield was found to fall when the dilution rate increased to 2 h^{-1}. The interaction between immobilized cells and medium was an important factor affecting the performance of the reactor. When the reactor was operated at a low flow rate, the solvent concentration around the cells' micro-environment was increased because of poor mass transfer. The increased solvent concentration inhibited the growth and metabolism of the cells, which led to low productivity and an excessive emission of by-products: CO_2 and H_2. The generation of by-products reduced the yield of glucose and caused a gas-hold problem, which further reduced mass transfer. The reactor experienced an increased solvent productivity with the vanishing of gas-hold and improvement of mass transfer when the dilution rate was set at 1 h^{-1}. If the dilution rate was set too high, say at 2 h^{-1}, the productivity was observed at a lower number. The decrease in productivity may have been due to excessive biomass growth and the increasing acid production activity.

The change of total acid concentration was found to be relatively flat, with the total acid concentration varying between 1.4 and 1.8 g l^{-1} with a dilution rate from 0.2 to 1 h^{-1}. However, increasing the dilution rate from 0.2 to 1 h^{-1} resulted in a dramatic increase in butyric acid concentration from 0.67 to 1.09 g l^{-1}, while a further increase from 1 to 2 h^{-1} resulted in a decrease in concentration to 0.74 g l^{-1}. The indistinctive change in acid concentration suggested that the immobilized cells have a shorter

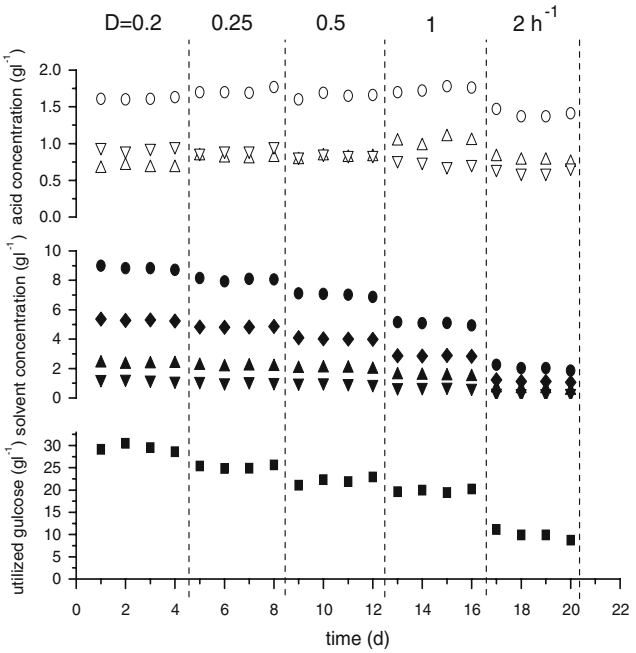

Fig. 2 Effect of dilution rate on the solvents and acid production, and glucose utilization during continuous fermentation of *C. beijerinckii* ATCC 55025. *Filled square* utilized glucose, *filled circle* total solvent, *filled triangle* acetone, *filled inverted triangle* ethanol, *filled diamond* butanol, *open circle* total acid, *open inverted triangle* butyric acid, *open triangle* acetic acid

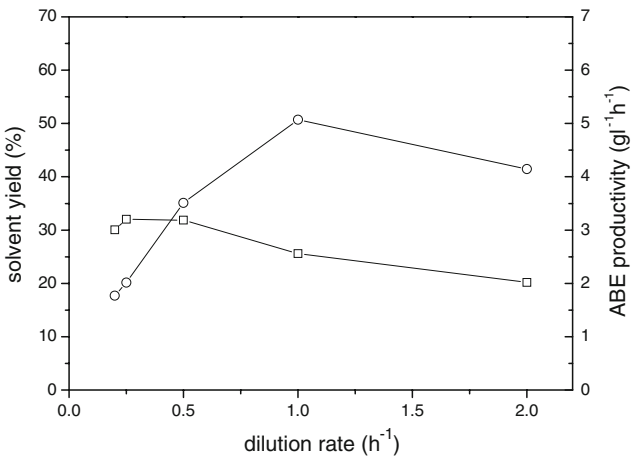

Fig. 3 Effect of dilution rate on the solvent yield and ABE productivity. *Open square* solvent yield, *open circle* ABE productivity

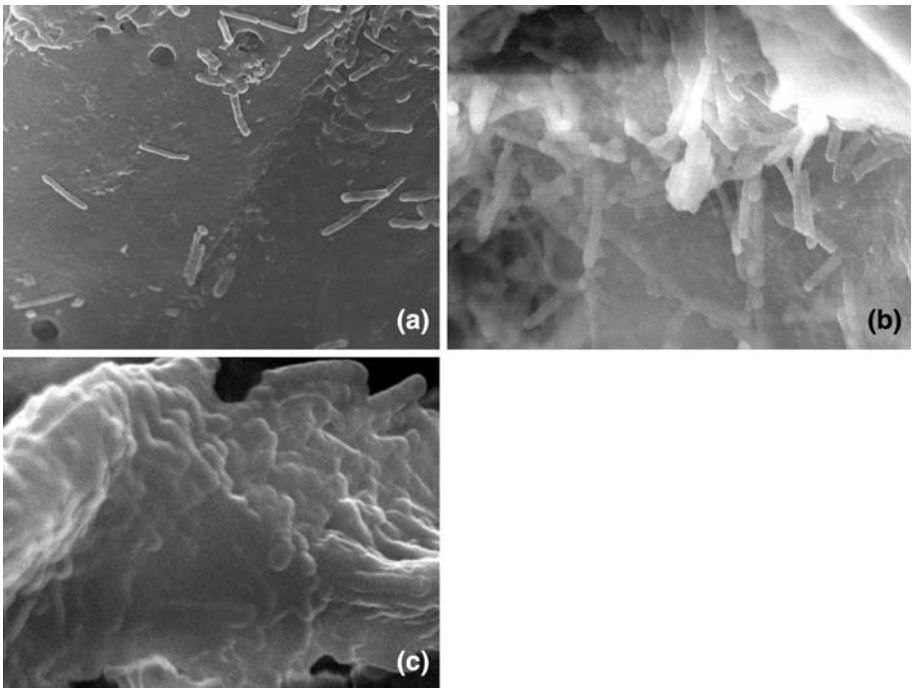

Fig. 4 Scanning electron micrograph of corn stalk with immobilized *C. beijerinckii* ATCC 55025. **a** The adsorbed cells adhere to the surface of the corn stalk at the beginning of the experiment, magnification ×2,000. **b** A large amount of cells has been observed at the end of the experiment, magnification ×2,000. **c** Dead cells were present in the reactor, magnification ×4,000

acidogenesis stage compared to the traditional ABE fermentation, whose acidogenesis stage is about 4 h [16]. Adding butyric acid to the medium resulted in a higher butanol concentration and a higher glucose-to-solvent yield [9, 14]. The efficiency of immobilized cells utilizing butyric acid pre-existing in the medium will be further studied.

The reactor was run at steady state for 20 days for this study. The steady state was then interrupted by the excessive growth of biomass. A mass of living and dead microbes blocked the reactor, which can be seen in Fig. 4b,c. The excessive growth of biomass was more obvious at a high dilution rate. In a future study, it would be interesting to see if limiting nutrition in the medium or keeping the cells in the starvation condition would extend the steady-state operation duration.

The performance of ABE fermentation with various immobilized cells is shown in Table 1. The data shown in the table suggest that the surface area of the support material has a significant impact on the performance of the immobilized cell reactor in terms of total solvent production and productivity of the operation.

Table 1 Comparison of continuous reactor performance with different cell immobilization techniques

Immobilized cell systems	Total solvents ($g\ l^{-1}$)	Productivity ($g\ l^{-1}\ h^{-1}$)	Yield ($g\ g^{-1}$)
Free cells[a] [6]	9.4	0.22	0.24
Polyvinyl alcohol[a] [6]	22.1	0.40	0.44
Brick [9]	7.9	15.80	0.38
Bonechar [7, 8]	6.5	6.50	0.38
Cotton towel[a] [13]	8.5	7.60	0.53
Carrageenan [1]	4.0	2.80	0.18
Chitosan [2]	2.7	1.43	0.18
Corn stalk (this work)	5.1	5.06	0.32

[a] With butyric acid pre-existing in the medium

Conclusions

A high solvent productivity of 5.06 $g\ l^{-1}\ h^{-1}$ was achieved when the corn stalk was used as a cell immobilization support. The top solvent productivity was achieved at a dilution rate of 1.0 h^{-1}, and the highest solvent yield was achieved at 0.25 h^{-1}. The dilution rate had a significant effect on the total solvent production, productivity, and yield, while its impact on total acid concentration was minimal. The reactor was operated at steady state for 20 days in continuous flow mode. The growth nutrients seem to need to be controlled for an extended stable state.

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