0273-2289/03/105-108/0375/$20.00

Continuous Production of Butanol from Starch-Based Packing Peanuts

Thaddeus C. Ezeji, Marisa Groberg, Nasib Qureshi,* and Hans P. Blaschek

University of Illinois, Biotechnology and Bioengineering Group, Department of Food Science and Human Nutrition, 1207 W. Gregory Drive, Urbana, IL 61801, E-mail: nqureshi@uiuc.edu

Abstract

Acetone, butanol, ethanol (ABE, or solvents) were produced from starch-based packing peanuts in batch and continuous reactors. In a batch reactor, 18.9 g/L of total ABE was produced from 80 g/L packing peanuts in 110 h of fermentation. The initial and final starch concentrations were 69.6 and 11.1 g/L, respectively. In this fermentation, ABE yield and productivity of 0.32 and 0.17 g/(L·h) were obtained, respectively. Compared to the batch fermentation, continuous fermentation of 40 g/L of starch-based packing peanuts in P2 medium resulted in a maximum solvent production of 8.4 g/L at a dilution rate of 0.033 h^{-1}. This resulted in a productivity of 0.27 g/(L·h). However, the reactor was not stable and fermentation deteriorated with time. Continuous fermentation of 35 g/L of starch solution resulted in a similar performance. These studies were performed in a vertical column reactor using *Clostridium beijerinckii* BA101 and P2 medium. It is anticipated that prolonged exposure of culture to acrylamide, which is formed during boiling/autoclaving of starch, affects the fermentation negatively.

Index Entries: Starch-based packing peanuts; continuous fermentation; butanol; *Clostridium beijerinckii* BA101; reactor; dilution rate.

Introduction

As a result of increasing oil prices (two to four times higher than in July–August 2000), and constant instability in the oil supply region (Middle East), various bioconversion programs have been initiated to produce biochemicals and bioenergy in the United States. To hasten research and development and find a solution to this crisis, President William J. Clinton

*Author to whom all correspondence and reprint requests should be addressed.

issued Executive Order 13134, entitled "Developing and Promoting Biobased Products and Bioenergy" (64 FR 44639, August 16, 1999). A primary goal of the order is to triple the nation's use of biobased products and bioenergy by 2010, generating as much as $20 billion annually in new income for farmers and rural communities.

As a result of these initiatives, we intensified our research on butanol fermentation. Butanol, which is an excellent biofuel, has numerous other applications in food, plastics, and chemical industries *(1)* and has been studied by numerous investigators *(2–6)*. In a recent economic study, we found that fermentation substrate is one of the most important factors that influence the price of butanol *(7)*. For that reason, waste substrates must be used for conversion to fuels and chemicals such as butanol. Starch-based waste packing peanuts, which were developed to biodegrade after use, are one such substrate and can be used for this bioconversion process. Successful bioconversion of such wastes would not only convert these waste substrates to useful chemicals, but also solves waste disposal and environmental problems faced by the relevant industries. *Clostridium beijerinckii* BA101, which was developed a few years ago, is capable of efficient starch hydrolysis in addition to superior production of solvents.

Fermentation studies using such wastes can be performed in either batch or continuous reactors. Since continuous reactors offer comparatively high reactor productivities, we chose to produce acetone, butanol, ethanol (ABE, or solvents) in these reactors. Hence, our objective was to produce ABE from starch-based packing peanuts in continuous reactors and compare reactor productivity with that of batch reactors.

Materials and Methods

Microorganism

A stock culture of *C. beijerinckii* BA101 was maintained as a spore suspension in distilled water at 4°C. Spores were heat shocked in cooked meat medium (Difco, Detroit, MI) containing 30 g/L of glucose at 80°C for 10 min. The culture was found to be growing actively within 16–18 h. This was followed by transferring 5 mL of actively growing culture to 100 mL of tryptone-glucose-yeast extract (TGY) medium *(8)*. Cells were grown anaerobically for 16–18 h at 34°C before being transferred into solvent production medium containing starch and/or packing peanuts.

Preparation of Medium

Continuous butanol production was studied from starch or starch-based packing peanuts (average volume of 67.57 cm^3/g, starch content of 88.4 wt%, average diameter of 1.54 cm, length of 2 to 3 cm; Storopack, Cleveland, OH). To prepare the medium for continuous fermentation, 35–40 g/L of starch or packing peanuts and 1 g/L of yeast extract (Difco) were dissolved in distilled water followed by sterilizing at 121°C for 15 min. The

medium was cooled by sweeping O_2-free N_2 gas across the surface. On cooling to room temperature, filter-sterilized P2 medium stock solutions *(9)* were added (10 mL of each of these solutions to 970 mL of autoclaved medium containing substrate and yeast extract):

1. Buffer: 50 g/L of KH_2PO_4, 50 g/L of K_2HPO_4, 220 g/L of ammonium acetate.
2. Vitamin: 0.1 g/L of paraaminobenzoic acid, 0.1 g/L of thiamin, 0.001 g/L biotin.
3. Mineral: 20 g/L of $MgSO_4 \cdot 7H_2O$, 1g/L of $MnSO_4 \cdot H_2O$, 1g/L of $FeSO_4 \cdot 7H_2O$, 1 g/L of NaCl.

The stock solutions were filter sterilized through a 0.2-µm-pore-size polyethersulfone membrane filter unit (Nalgene, Rochester, NY). The medium was kept at room temperature and stirred at <100 rpm to avoid any sedimentation. The medium was kept in an airtight sealed bottle provided with an inlet and outlet for O_2-free N_2 gas to keep it anaerobic. The gas was swept across the surface of the medium at all times. Starch or packing peanut concentration was 35–40 g/L to keep feed viscosity low and to be able to pump the feed through the pump tube.

For batch fermentation, the medium was prepared in a 500 to 1000-mL beaker followed by transferring to 150-mL screw-capped bottles (125 mL of medium). The medium contained starch or packing peanuts at 60–80 g/L, yeast extract at 1 g/L, and was autoclaved in bottles. On cooling, the stock solutions were added to the bottles followed by transferring them to an anaerobic chamber (Coy, Ann Arbor, MI) for 24 h for anaerobiosis. This was followed by inoculation with actively growing TGY cell suspension. Fermentation proceeded at 35°C until complete and 1-mL samples were collected intermittently. The samples, from both the bottles and the reactor, were centrifuged at 14,000 rpm in Eppendorf centrifuge tubes before injecting the supernatant into the gas chromatograph for solvent analysis.

Note that pH was not controlled in any of the fermentations. However, in the case of batch fermentation (starch or packing peanuts), it was adjusted automatically to approx 6.8 by the addition of buffer solution. Such a high pH favors quick growth and acid production, which lowers pH to approx 5.0 and triggers solvent production. In addition to temperature and pH control, maintaining anaerobic conditions in the incubator is a prerequisite to a successful butanol fermentation using *C. beijerinckii* BA101.

Continuous Reactor

The reactor was composed of a 312-mL total volume jacketed polyacrylic column (192 × 46 mm) and was used in vertical mode. The reactor was sterilized with 30% (v/v) ethanol solution for 48-72 h, after which it was drained and washed thoroughly with sterilized deionized water. O_2-free N_2 gas was passed through the column overnight to ensure anaerobic conditions inside the reactor. Twenty milliliters of actively growing *C. beijerinckii* BA101 cells from a TGY bottle was inoculated into the reactor,

and the reactor was filled with the starch/packing peanut–based fresh P2 medium described earlier. Cell growth was allowed inside the reactor for 6 h, after which the P2 medium was continuously fed using a peristaltic pump (Cole-Parmer, Veron Hills, IL) and silicone tubing. The reactor was fed at the bottom, thus getting effluent at the top. Since it was a free-cell reactor, feed rate was kept low for solventogenesis (Dilution rate [D, h^{-1}] < μ [specific growth rate constant, h^{-1}] and ν [specific rate of solvent production, h^{-1}]). Water at 35°C was circulated through the jacket of the column to control temperature using a circulating water bath (Polystat; Cole-Parmer).

Analytical Procedures

Because of the opaque nature of starch and of peanut solutions, cell concentration measurement by optical density was not possible. 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). Batch fermentation productivity was calculated as total ABE concentration (g/L) divided by fermentation time (h). Fermentation time was defined as the time period when a maximum ABE concentration was reached. ABE yield, which does not have a unit, was calculated as total ABE produced (g) divided by total carbohydrate (as starch) utilized (g). In the continuous fermentation, productivity was calculated as ABE concentration (g/L) multiplied by dilution rate (h^{-1}). Dilution rate is defined as feed flow rate (mL/h) per reactor volume (mL).

Glucose concentration was determined using a hexokinase and glucose-6-phosphate dehydrogenase (Sigma, St. Louis, MO) coupled enzymatic assay. To measure glucose, 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 mg/mL of glucose in 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.

Starch concentration of the sample was determined using a modified method of Holm et al. *(10)*. A sample (250 mg) was suspended in distilled water (15 mL) in a 50-mL beaker. Heat-stable α-amylase (100 μL) (Sigma) was added and mixed gently with a magnetic stirrer. The beaker was placed in a boiling water bath for 30 min with mixing every 5 min. The suspension was allowed to cool under continuous agitation on a magnetic stirrer and was transferred to a 25-mL volumetric flask followed by filling

it with water to the volume. One milliliter of the solution was transferred to a test tube followed by the addition of 2.9 mL of 0.1 *M* sodium acetate buffer (pH 4.75) and 100 µL of amyloglucosidase (Sigma). The mixture was incubated for 60 min at 55°C with careful mixing every 5 min. The sample was then transferred to a 50-mL volumetric flask and filled to volume with distilled water. Ten microliters of the solution was assayed for glucose according to the hexokinase and glucose-6-phosphate dehydrogenase assay method as described earlier.

$$\% \text{ starch} = (\text{mg glucose} \times 25^a \times 50^a \times 0.9^b \times 100)/(\text{Sample weight [250 mg]})$$

in which a is the dilution factor and b is the correction glucose → glucan.

At the time of these studies, it was not possible to analyze starch-based packing peanuts for all of their constituent components.

Results and Discussion

To evaluate fermentability of packing peanuts, a batch fermentation was run with 80 g/L of packing peanuts using *C. beijerinckii* BA101 in P2 medium. The initial and final concentrations of starch were 69.6 and 11.1 g/L, respectively *(11)*. This resulted in utilization of 58.5 g/L of starch and a solvent yield of 0.32. During 110 h of fermentation, 18.9 g/L of ABE (losses not included) was produced, resulting in a productivity of 0.17 g/(L·h). The concentration of acids was low at 0.2 g/L at 110 h. The results proved that packing peanuts were successfully utilized by *C. beijerinckii* BA101 to produce ABE. However, productivity was low at 0.17 g/(L·h).

To improve reactor productivity, a continuous reactor was run at a flow rate of 10.2 mL/h (dilution rate of 0.033 h^{-1}). For the first 121 h, fermentation was vigorous but then slowed down. After a period of 48 h of continuous operation, 6.7 g/L of total ABE was obtained in the effluent, and it continued to increase to 8.4 g/L at 83 h (Table 1). At 121 h, ABE concentration was 8.3 g/L, with a maximum productivity of 0.27 g/(L·h). After 121 h of operation, the fermentation started deteriorating constantly, as shown in the Table 1. Fermentation was continued for 246 h, and at that time 2.3 g/L of total ABE and 5.7 g/L of total acids were present in the effluent. At that stage, significant amount of sediments had accumulated in the bottom part of the reactor (up to 6.5 cm high). It was thought that accumulated sediments containing undisclosed chemicals present in the packing peanuts had affected the fermentation negatively. A second reactor run under identical conditions produced similar results.

To check if fermentation efficiency was reduced owing to suspension, reactor liquid was drained into a separating funnel and sediments were separated. In addition, the volume of sediments was madeup to 312 mL (equal to the liquid volume in the reactor) by adding distilled water. To this 18.7 g of glucose (60 g/L) and 0.3 g of yeast extract (1 g/L) were added followed by autoclaving. On cooling, P2 medium stock solutions were added. The mixture was fermented in 150-mL screw-capped bottles inocu-

Table 1
Continuous Production of ABE
from Starch-Based Packing Peanuts Using *C. beijerinckii* BA101

Time (h)	Products (g/L)						ABE productivity (g/(L·h))
	Acetone	Butanol	Ethanol	Acetic acid	Butyric acid	Total ABE	
24	0.9	2.8	0.1	2.7	1.1	3.8	0.12
48	1.7	4.9	0.1	2.3	1.0	6.7	0.22
72	1.6	6.4	0.1	1.6	1.1	8.1	0.26
83	1.7	6.6	0.1	1.3	0.9	8.4	0.27
102	1.4	5.7	0.1	1.8	0.8	7.2	0.24
121	1.7	6.5	0.1	1.4	1.3	8.3	0.27
143	1.0	4.2	0.1	1.9	1.3	5.3	0.17
151	1.1	4.7	0.1	1.8	2.2	5.9	0.19
169	1.0	2.8	0.1	3.0	1.5	3.9	0.13
175	0.7	2.1	0.1	1.9	2.1	2.9	0.09
196	0.7	2.2	0.1	2.2	2.3	3.0	0.09
216	0.7	2.1	0.1	3.7	3.3	2.9	0.09
225	0.8	2.2	0.1	3.5	2.2	3.1	0.10
241	0.6	1.8	0.0	3.5	2.2	2.4	0.08
246	0.6	1.7	0.0	3.5	2.2	2.3	0.08

lated with freshly grown inoculum. In 96 h of fermentation, 14.5 g/L of total ABE and 5.2 g/L of total acids were produced, suggesting that the sediments were not toxic to the fermentation in the reactor (Fig. 1).

To further strengthen these findings, a continuous reactor was operated with 35 g/L of starch solution in feed and P2 medium ingredients. The continuous reactor was operated under identical conditions (dilution rate of 0.033 h^{-1}). The performance of the reactor was similar to that of the previous reactors fed with packing peanuts solution. At 46 h of continuous operation, 8.1 g/L of total ABE and 2.1 g/L of total acids were produced (Table 2). For 103 h the reactor was productive and produced up to 8.5 g/L of ABE, resulting in a productivity of 0.28 g/(L·h). Although this productivity is higher than achieved in the batch reactor, the reactor was not steady. At 226 h, 3.7 g/L of ABE and 5.5 g/L of acids were present in the reactor effluent. In this experiment, a similar type of sediment was also observed at the bottom of the reactor. However, the previous batch experiment suggested that sediments were not toxic to the culture.

A recent study reported that formation of acrylamide results from boiling of the starch solution (personal communication). It is well known that acrylamide inhibits microbial cell growth and activity. Hence, it was possible that acrylamide produced during boiling and autoclaving of the starch-based packing peanuts/starch solution was inhibitory to *C. beijerinckii* BA101 cells. Possibly, prolonged exposure inhibited the cul-

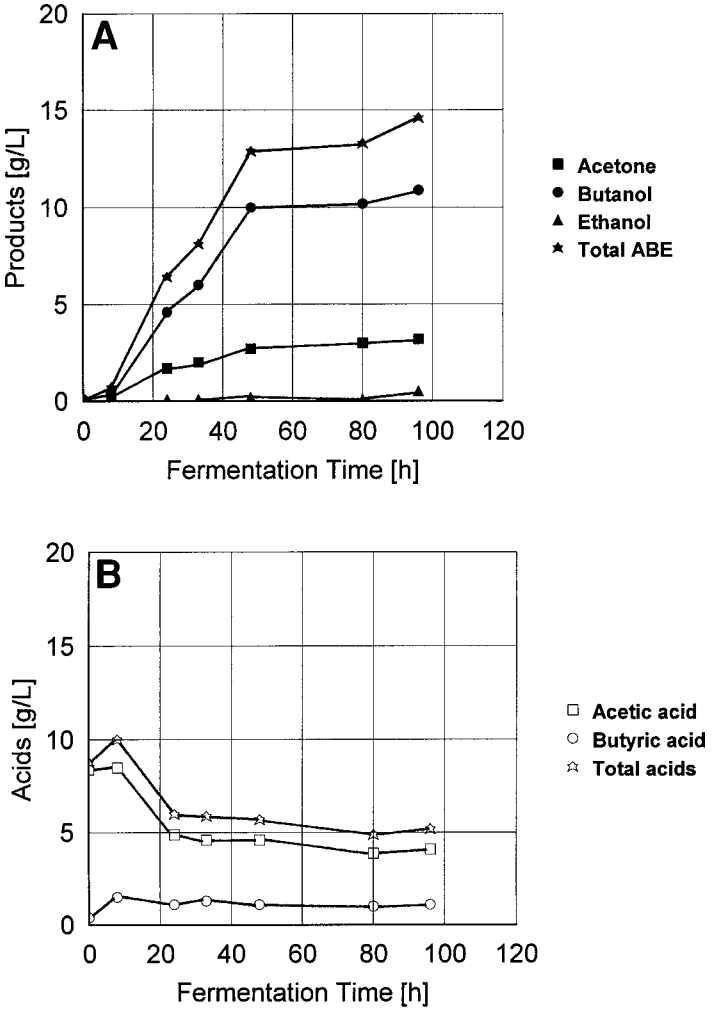

Fig. 1. Effect of packing peanut sediments on butanol fermentation using *C. beijerinckii* BA101 and P2 medium. (**A**) Solvents; (**B**) acids.

ture. At this stage, we conclude that batch fermentation of packing peanuts was successful. However, continuous fermentation of starch/packing peanuts, which always had a high initial productivity, deteriorated after approx 120 h of operation.

Acknowledgments

We gratefully acknowledge the kind gift of the packing peanuts from Storopack, Cleveland, OH. This work was supported by grants from Illinois Corn Marketing Board and Illinois Council on Food and Agricultural Research (CFAR IDA CF 01E-35-1).

Table 2
Continuous Production of ABE from 35 g/L of Starch Using *C. beijerinckii* BA101

Time (h)	Products (g/L)						ABE productivity (g/(L·h))	Glucose (g/L)[a]
	Acetone	Butanol	Ethanol	Acetic acid	Butyric acid	Total ABE		
24	0.8	2.4	0.1	2.5	1.4	3.3	0.11	1.26
31	0.9	2.9	0.1	2.2	1.3	3.9	0.13	0.89
46	2.5	5.4	0.2	1.7	0.4	8.1	0.26	0.03
51	2.5	5.7	0.2	1.2	0.3	8.4	0.27	0.07
70	2.3	5.7	0.2	1.6	0.8	8.2	0.27	0.04
77	2.4	5.5	0.2	1.4	0.5	8.1	0.26	0.04
91	2.4	5.9	0.2	1.6	0.5	8.5	0.28	0.05
103	2.3	5.8	0.2	2.1	0.7	8.3	0.27	0.05
114	2.1	5.5	0.1	2.8	0.9	7.7	0.25	0.08
124	1.8	5.0	0.1	2.3	0.8	6.9	0.23	0.06
139	1.8	4.9	0.1	2.9	1.1	6.8	0.22	0.07
150	1.6	4.5	0.2	2.9	1.1	6.3	0.21	0.21
163	1.5	4.1	0.1	3.1	1.3	5.7	0.19	0.20
170	0.8	2.7	0.1	3.5	1.1	3.6	0.12	0.62
188	1.0	2.6	0.1	3.5	1.5	3.7	0.12	0.38
196	1.0	2.6	0.1	3.8	1.5	3.7	0.12	0.31
211	1.0	2.6	0.1	3.6	1.6	3.7	0.12	0.21
226	0.8	2.8	0.1	3.7	1.8	3.7	0.12	0.04

[a]In reactor effluent.

References

1. Qureshi, N. and Blaschek, H. P. (2001), *J. Ind. Microbiol. Biotechnol.* **27,** 292–297.
2. Davison, B. H. and Scott, C. D. (1988), *Appl. Biochem. Biotechnol.* **18,** 19–34.
3. Davison, B. H. and Thompson, J. E. (1993), *Appl. Biochem. Biotechnol.* **39,** 415–425.
4. Friedl, A., Qureshi, N., and Maddox, I. S. (1991), *Biotechnol. Bioeng.* **38,** 518–527.
5. Ennis, B. M., Maddox, I. S., and Schoutens, G. H. (1986), *NZJ Dairy Sci. Technol.* **21,** 99–109.
6. Chen, C. K. and Blaschek, H. P. (1999), *Appl. Microbiol. Biotechnol.* **52,** 170–173.
7. Qureshi, N. and Blaschek, H. P. (2000), *Trans IChemE* **78(Part C),** 139–144.
8. Formanek, J., Mackie, R., and Blaschek, H. P. (1997), *Appl. Environ. Microbiol.* **63,** 2306–2310.
9. Qureshi, N. and Blaschek, H. P. (1999), *Biomass Bioenergy* **17,** 175–184.
10. Holm, J., Bjorck, I., Drews, A., and Asp, N.-G. (1986), *Starch/Starke* **38,** 224–226.
11. Jesse, T. W., Ezeji, T. C., Qureshi, N., and Blaschek, H. P. (2002) *J. Ind. Microbiol. Biotechnol.* **29,** 117–123.