Journal of Industrial Microbiology & Biotechnology (1998) 21, 187–191
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Development of a cost-effective glucose-corn steep medium for production of butanol by *Clostridium beijerinckii*

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Corn steep water (CSW) medium (1.6% solids plus 6% glucose) was evaluated for growth and butanol production by *Clostridium beijerinckii* NCIMB 8052 wild-type and hyper-amylolytic, hyper-butanol-producing mutant strain BA101. CSW alone was not a suitable substrate, whereas addition of glucose supported growth and butanol production by both strains. In a batch-scale fermentation using an optimized 6% glucose-1.6% solids CSW medium, *C. beijerinckii* NCIMB 8052 and strain BA101 produced 10.7 g L^{-1} and 14.5 g L^{-1} of butanol, respectively. The total solvents (acetone, butanol, and ethanol) produced by *C. beijerinckii* NCIMB 8052 and strain BA101 were 14 g L^{-1} and 20 g L^{-1}, respectively. Initial fermentation in small-scale flasks containing 6% maltodextrin-1.6% solids concentration CSW medium resulted in 6 g L^{-1} and 12.6 g L^{-1} of butanol production by *C. beijerinckii* NCIMB 8052 and strain BA101, respectively. CSW can serve as an economic source of nitrogen, vitamins, amino acids, minerals, and other nutrients. Thus, it is feasible to use 6% glucose-1.6% solids CSW medium in place of semi-defined P2 medium.

Keywords: *Clostridium beijerinckii*; butanol; solvent production; corn steep water

Introduction

The production of solvents, namely acetone, butanol, and ethanol by clostridia is well known [7,11]. There is a renewed interest in production of butanol using fermentation by clostridia. Butanol is extensively used as a feedstock chemical. Currently, it is derived from petrochemical processes. The current US market for butanol is 1.6 billion kg which is expected to grow 4% annually. However, the uncertainty associated with petrochemical supplies has caused a resurgence in interest in the traditional acetone-butanol-ethanol (ABE) fermentation using the clostridia. Unfortunately, the butanol fermentation utilizing *Clostridium* strains suffers from the high cost of solvent recovery due to the low final butanol concentration in the fermentation broth, and the high cost of medium components. We have investigated the use of corn steep water (CSW) as an inexpensive source of nutrients for development of an economical medium for production of butanol by *Clostridium beijerinckii* NCIMB 8052 parent strain and strain BA101, a hyper-amylolytic, hyper-butanol-producing mutant strain.

CSW is a by-product of the corn wet-milling industry. Currently, CSW is evaporated to a 50% solids syrup (corn steep liquor) that has been marketed primarily as an animal feed supplement in the cattle industry at $55 per ton [13]. CSW has also been used as a nutrient source for production of ethanol by yeast [1,8,12], and *E. coli* [9], and acetate by *Clostridium thermoaceticum* [3,14]. Previously our laboratory reported enhanced production of butanol by *C. beijerinckii* BA101 in a semidefined P2 medium containing glucose, yeast extract, vitamins, minerals, and buffers [4]. Since CSW contains a rich complement of important nutrients such as amino acids, vitamins, nitrogen and minerals [6], this study focused on development of CSW medium for growth and butanol production by *C. beijerinckii* strains NCIMB 8052 and BA101.

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Received 9 February 1998; accepted 1 September 1998

Materials and methods

Microorganisms

C. beijerinckii strains NCIMB 8052 and BA101 were used in this study. Stock cultures were maintained as spores in cooked meat medium at 4°C. Spores were heat-shocked at 80°C for 10 min, and were grown anaerobically in TGY (tryptone-glucose-yeast extract) medium at 37°C.

Medium

Glucose-CSW medium contained various concentrations of glucose and CSW. In addition, glucose-CSW medium contained 0.1% cysteine-HCl as a reducing agent. For preparation of glucose-CSW medium, 10% solids CSW was pre-treated as follows. To raw CSW (pH 4.2), cysteine-HCl was added followed by adjustment of the pH to 6.8. The CSW was left at 0°C overnight, and was centrifuged the following day at 27 500 × *g* for 60 min at 4°C. The clear CSW supernatant obtained after centrifugation was diluted with distilled water to achieve the desired solids concentration. The diluted CSW was then supplemented with an appropriate amount of glucose, and filter-sterilized using a sterile 0.2-μm cellulose nitrate filter unit (Nalge Co, Rochester, NY, USA) in preparation for fermentation studies. A single batch of CSW was used for each experiment.

Batch fermentation

Fermentation studies were conducted at the 500-ml level using a Multigen fermentor (New Brunswick Scientific Co,

Edison, NJ, USA) at 30°C. The medium was inoculated with a 5% (vol/vol) inoculum, and was continuously bubbled with 50 ml min^{-1} of N_2. Glucose-CSW medium inoculated with TGY culture was used as an inoculum. Antifoam 289 (Sigma Chemical Co, St Louis, MO, USA) was added as necessary to control foam in the fermentor.

Analytical methods

Cell growth was evaluated by measuring optical density at 600 nm using a DU®-40 Spectrophotometer (Beckman Instruments, Fullerton, CA, USA). For product analysis, the sample was centrifuged in a microfuge and the supernatant phase was frozen until analyzed. Solvents and acids were analyzed using a gas chromatograph (5710A; Hewlett-Packard Co, Avondale, PA, USA) equipped with a flame ionization detector and a glass column (1.83 m × 2 mm i.d.) packed with 80/100 Carbopack C-0.1% SP-1000 (Supelco, Bellefonte, PA, USA). Run conditions used were the same as previously reported [4].

Results and discussion

Effect of sterilization and pH

The pH of raw CSW is about pH 4.2, and it is believed to contain a high number of microorganisms such as lactic acid bacteria. It was necessary to first examine the suitability of various sterilization methods of raw CSW on growth of *C. beijerinckii* BA101. Autoclaving or filtration of raw CSW was employed as a means of sterilization. Either autoclaved or filtered CSW at pH 4.5 served as a poor substrate for growth of *C. beijerinckii* BA101.

When the pH of raw CSW was adjusted from 4.2 to 6.5, only filter-sterilized CSW was able to support limited growth of *C. beijerinckii* BA101. Addition of vitamins (*p*-aminobenzoic acid, thiamine, and biotin) to the autoclaved CSW at pH 6.5 allowed growth of *C. beijerinckii* BA101 which was similar to that for filter-sterilized CSW, suggesting that autoclaving may have destroyed essential vitamins and other growth factors. These results indicate that the method of sterilization used along with the final pH of CSW can have an effect on the growth of *C. beijerinckii* BA101. However, growth and the final butanol concentration produced by *C. beijerinckii* BA101 in the undiluted and unsupplemented CSW at pH 6.5 were low.

Effect of CSW dilution and glucose supplementation

The raw undiluted CSW that was collected from the wet-milling process typically contained 10% solids. The next step was to examine the effect of supplementing undiluted CSW (pH 6.5) with various concentrations of glucose on growth and butanol production by *C. beijerinckii* BA101. After the first transfer of *C. beijerinckii* BA101 from a TGY tube, the OD 0.4 was observed in medium without CSW *vs* OD 3.9 in medium with CSW. When a second transfer was made into the same medium, no growth was seen in medium without CSW, whereas OD 1.9 was seen in medium containing CSW. The small amount of growth seen in medium without CSW after the first transfer may have been due to carry-over of nutrients from TGY medium. These results suggest that addition of CSW to medium containing dextrose is necessary for the growth of *C. beijerinckii* BA101. As shown in Table 1, the growth of *C. beijerinckii* BA101 in 10% solids concentration CSW decreased with increasing glucose levels. Although butanol production was obtained in undiluted CSW supplemented with 2% glucose, the growth and butanol production by *C. beijerinckii* BA101 in this medium was far below the levels obtained with semi-defined P2 medium [4]. Therefore, various dilutions of CSW supplemented with different concentrations of glucose were examined in order to find the best combination of CSW and glucose for maximal growth and butanol production by *C. beijerinckii* BA101 (Table 1). CSW diluted to 1.25% solids supplemented with 5% glucose was able to support good growth, and allowed butanol production at a level of 8.8 g L^{-1} by *C. beijerinckii* BA101. Further optimization studies revealed that 1.6% solids concentration CSW plus 6% glucose was optimal for butanol production by *C. beijerinckii* BA101 and resulted in a 17% higher butanol concentration relative to the 1.25% solids concentration CSW plus 5% glucose medium.

Effect of addition of other nutrients

A series of experiments was performed in order to further optimize the 6% glucose-1.6% solids CSW medium for growth and butanol production by *C. beijerinckii* BA101 by using semi-defined P2 medium as a model. P2 medium consisting of glucose, yeast extract, vitamins, minerals, and buffers has been successfully used for growth and solvent production by *C. beijerinckii* BA101 [4]. In addition to amino acids, heavy metals, and vitamins, CSW also contains a complex mixture of carbohydrates, peptides, various organic compounds (lactic acid, glycolic acid, phenolic

Table 1 Effect of CSW and glucose concentrations on growth and solvent production by *C. beijerinckii* BA101[a]

CSW solids concentration (g per 100 ml)	Glucose added (%)	Optical density (600 nm)	Butanol concentration (g L^{-1})
10.0	0	1.8	3.9
	1	1.8	4.1
	3	1.3	6.3
	4	1.4	6.9
	5	1.0	7.4
	6	1.4	7.1
5.0	2	1.5	3.2
	3	1.5	3.8
	4	3.1	3.5
	5	3.2	5.3
	6	3.3	5.8
	7	2.5	5.2
2.5	2	2.7	4.8
	3	2.8	6.9
	4	2.1	6.2
	5	2.0	6.8
	6	2.0	6.9
	7	1.9	6.9
1.25	2	2.8	5.1
	3	3.1	6.2
	4	3.1	7.5
	5	3.2	8.8
	6	3.3	8.6
	7	2.5	7.4

[a] Growth and butanol concentrations were measured after 48 h of incubation.

Table 2 Effect of nutrient supplementation of 6% glucose-1.6% solids CSW medium on growth and solvent production by *C. beijerinckii* BA101[a]

Nutrient added	Optical density (600 nm)	Butanol produced (g L^{-1})	Butanol/acetone ratio
None	2.5	11.7	1.5
Vitamins[b]	2.6	12.1	1.5
Minerals[c]	2.4	14.9	2.9
Buffers[d]	2.2	10.4	1.6
Yeast extract[e]	2.6	11.8	1.5
Tryptone[e]	2.5	13.4	1.6

[a]Cells were grown in glucose-CSW medium for 99 h.
[b]Medium contained per liter: 1 mg *p*-aminobenzoic acid, 1 mg thiamine, and 0.01 mg biotin.
[c] Medium contained per liter: 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 0.01 g NaCl.
[d] Medium contained per liter: 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, and 2.2 g ammonium acetate.
[e] Medium contained per liter: 20 g yeast extract or 20 g tryptone.

acids, amines, alcohols, and fatty acids), inorganic ions, *myo*-inositol phosphates, and SO_2 [5,6].

As shown in Table 2, various vitamins, minerals, buffers and sources of complex nutrients were added to the 6% glucose-1.6% solids CSW medium. Addition of vitamins or yeast extract or buffering the medium using KH_2PO_4 plus K_2HPO_4 plus NH_4-acetate did not stimulate growth or butanol production by *C. beijerinckii* BA101. However, addition of tryptone or minerals resulted in 14% and 27% increase in butanol levels by *C. beijerinckii* BA101, respectively. The ratio of butanol to acetone was also highest when minerals were added to the 6% glucose-1.6% solids CSW medium.

Further studies on the effect of individual minerals were carried out with *C. beijerinckii* BA101. As shown in Table 3, NaCl, $MnSO_4 \cdot H_2O$, and $MgSO_4 \cdot 7H_2O$ had no effect on growth or butanol production. However, $FeSO_4 \cdot 7H_2O$ stimulated both growth and butanol production. Addition of iron to a 6% glucose-1.6% solids CSW medium resulted in a 26% increase in butanol production, and the ratio of butanol to acetone was also higher than that of the culture without added iron.

The experiments regarding effect of mineral and tryptone supplementation (Table 2) and iron effect (Table 3) have been repeated. We have consistently observed that mineral and tryptone supplementation stimulated butanol production by 27.5% (± 0.5%) and 13% (± 1%), respectively. Iron also stimulated butanol production by 25% (± 1%).

Table 3 Effect of mineral supplementation of 6% glucose-1.6% solids CSW medium on growth and solvent production by *C. beijerinckii* BA101[a]

Mineral salt added[b]	Optical density (600 nm)	Butanol produced (g L^{-1})	Butanol/acetone ratio
None	2.4	10.3	2.9
NaCl	2.6	10.0	3.0
$FeSO_4 \cdot 7H_2O$	3.2	13.0	3.9
$MnSO_4 \cdot H_2O$	2.3	9.0	3.5
$MgSO_4 \cdot 7H_2O$	2.5	8.5	3.9
All of the above	2.9	13.2	4.0

[a] Cells were grown in glucose-CSW medium for 48 h.
[b] Medium contained minerals per liter when added: 0.2 g $MgSO_4 \cdot 7H_2O$, 0.01 g $MnSO_4 \cdot H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$ and 0.01 g NaCl.

Batch fermentation

The 6% glucose-1.6% solids CSW medium which was developed above was tested for growth and solvent production by *C. beijerinckii* strains NCIMB 8052 and BA101 in a batch fermentation. Figures 1 and 2 show cell growth, acids and solvents produced by *C. beijerinckii* strains 8052 and BA101, respectively. Both strains reached maximum growth after 48 h of incubation, and exhibited a doubling time of 8–9 h in this medium. Maximal butanol production of 10.7 g L^{-1} was attained after 90 h of cultivation for *C. beijerinckii* 8052. Acetone and ethanol produced by *C. beijerinckii* 8052 were at a concentration of 2.5 g L^{-1}

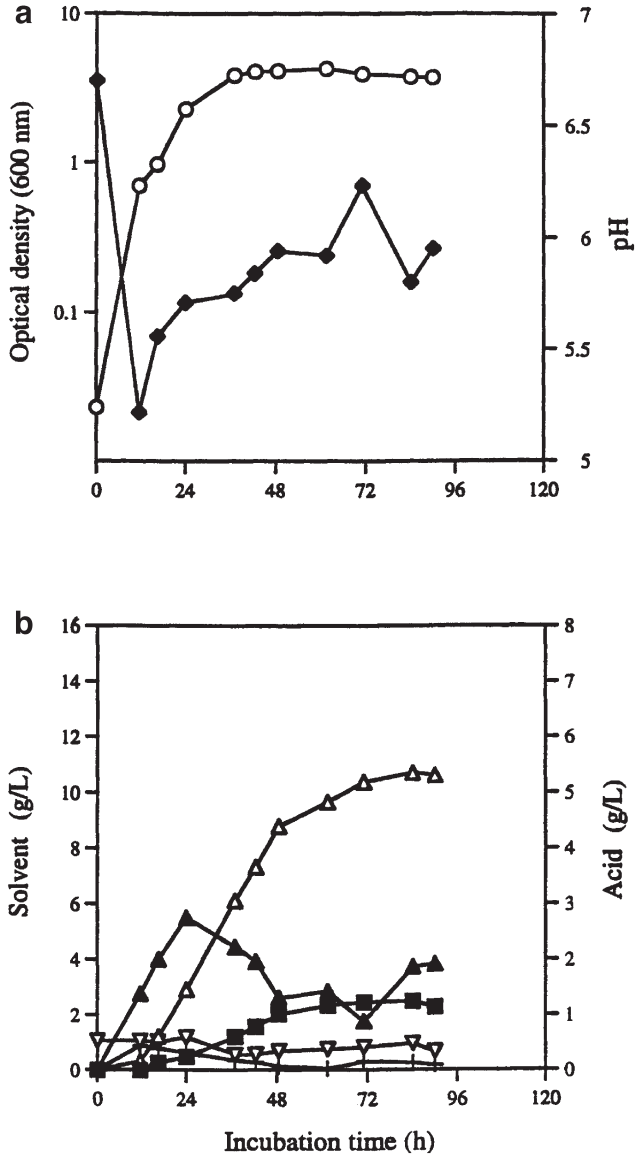

Figure 1 Growth, pH (a), and acid, solvent production (b) during 500 ml batch fermentation by *C. beijerinckii* NCIMB 8052 in 6% glucose-1.6% solids corn steep medium. Symbols: ○, optical density; ◆, pH; ■, acetone; △, butanol; ▽, ethanol; ▲, acetic acid; +, butyric acid.

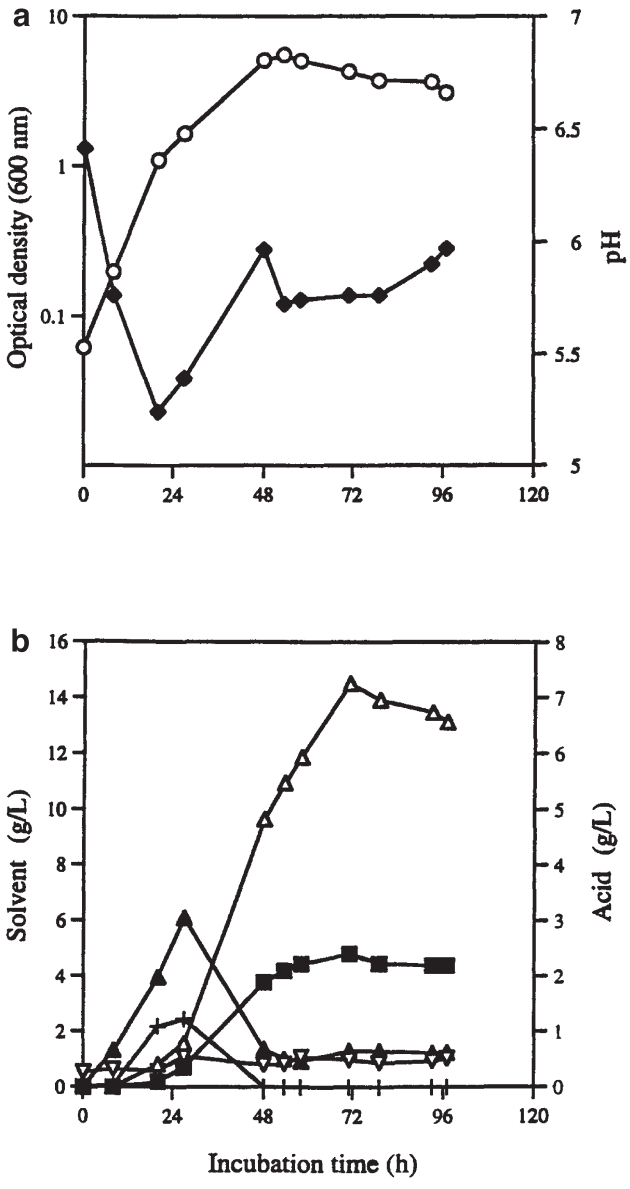

Figure 2 Growth, pH (a), and acid, solvent production (b) during 500 ml batch fermentation by *C. beijerinckii* BA101 in 6% glucose-1.6% solids corn steep medium. Symbols: ○, optical density; ◆, pH; ■, acetone; △, butanol; ▽, ethanol; ▲, acetic acid; +, butyric acid.

and 1 g L^{-1}, respectively. When *C. beijerinckii* BA101 was grown in 6% glucose-1.6% solids CSW medium, a maximal butanol concentration of 14.5 g L^{-1} was achieved after 72 h. *C. beijerinckii* BA101 also produced acetone and ethanol at a concentration of 4.8 g L^{-1} and 1 g L^{-1}, respectively. Compared to *C. beijerinckii* NCIMB 8052, strain BA101 produced 35% and 43% higher levels of butanol and total solvents in 6% glucose-1.6% solids CSW medium, respectively. The initial level of acetic acid produced was similar for both strains. In contrast, *C. beijerinckii* BA101 not only produced a higher level of butyric acid compared to *C. beijerinckii* NCIMB 8052, but was also able to utilize butyric acid completely during the fermentation. This observation may be the result of an enhanced capacity for uptake and recycling of butyric acid

Table 4 Growth and solvent production by *C. beijerinckii* strains NCIMB 8052 and BA101 with 6% glucose or maltodextrin as a substrate in 1.6% solids CSW medium[a]

Strain	Substrate	Optical density (600 nm)	Butanol produced (g L^{-1})
8052	Glucose	2.6	8.5
	Maltodextrin	3.2	6.0
BA101	Glucose	2.7	10.6
	Maltodextrin	4.0	12.6

[a] Cells were grown in CSW medium containing appropriate carbohydrate for 48 h.

by *C. beijerinckii* BA101 [4]. In a separate flask experiment containing 50 ml culture, additional glucose was added to the culture after 12 h of incubation in order to examine the role of glucose supplementation on solvent production. These additions of glucose did not improve growth or butanol production by *C. beijerinckii* BA101 relative to unsupplemented medium (data not shown). Similarly, addition of fresh CSW after 18 h of incubation did not improve either growth or butanol production by strain BA101.

Similar results for growth and solvent production by *C. beijerinckii* BA101 were obtained when using corn steep liquor derived from Sigma Chemical Company and Marcor Development Corp (Hackensack, NJ, USA). It should also be possible to replace glucose in glucose-CSW medium with other available substrates (eg, starch, fructose, xylose etc) for conversion to butanol. Table 4 compares growth and solvent production by both *C. beijerinckii* strains grown in a CSW medium containing either glucose or maltodextrin (produced from corn starch) as a carbohydrate source. Both strains grew better on maltodextrin when compared to glucose. In addition, *C. beijerinckii* BA101 produced 19% higher levels of butanol when grown on maltodextrin than on glucose.

Lemmel [10] used corn steep liquor in the growth medium of a solventogenic *Clostridium*. Our studies were primarily concerned with optimizing the medium for growth and solvent production by *C. beijerinckii* BA101, a hyper-amylolytic, hyper-butanol-producing strain [2]. Further studies in our laboratory are directed at scaling-up the glucose-CSW-based fermentation process, and using other carbohydrates such as starch with CSW for butanol production when using *C. beijerinckii* BA101.

Acknowledgements

The corn steep water was kindly provided by AE Staley Manufacturing Company (Decatur, IL, USA). This study was funded in part by Illinois Corn Marketing Board, and the Council on Food and Agricultural Research.

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