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Soy molasses as fermentation substrate for production of butanol using *Clostridium beijerinckii* BA101

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Spray-dried soy molasses (SDSM) contains the sugars dextrose, sucrose, fructose, pinitol, raffinose, verbascose, melibiose, and stachyose. Of the 746 g kg^{-1} total sugars in SDSM, 434 g kg^{-1} is fermentable using *Clostridium beijerinckii* BA101. SDSM was used to produce acetone, butanol, and ethanol (ABE) by *C. beijerinckii* BA101 in batch cultures. Using 80 g l^{-1} SDSM, 10.7 g l^{-1} ABE was produced in P2 medium. Higher concentrations of SDSM resulted in poor solvent production due to the presence of excessive salt and inhibitory components. *C. beijerinckii* BA101 in SDSM at 80 g l^{-1} concentration produced 22.8 g l^{-1} ABE when supplemented with 25.3 g l^{-1} glucose. SDSM contains 57.4 g kg^{-1} mineral ash and 2% tri-calcium phosphate. Tri-calcium phosphate up to 43.1 g l^{-1} was not inhibitory and at a tri-calcium phosphate concentration of 28.8 g l^{-1}, the culture produced more solvents (30.1 g l^{-1}) than the control experiment (23.8 g l^{-1}). In contrast, sodium chloride was a strong inhibitor of *C. beijerinckii* BA101 cell growth. At a concentration of 10 g l^{-1} sodium chloride, a maximum cell concentration of 0.6 g l^{-1} was achieved compared to 1.7 g l^{-1} in the control experiment. The effects of two salts on specific growth rate constant (μ) and specific rate of ABE production (ν) for *C. beijerinckii* BA101 were examined. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 290–295.

Keywords: SDSM; ABE; butanol; *C. beijerinckii* BA101; sodium chloride; tri-calcium phosphate; μ; ν

Introduction

Fluctuations in oil prices have resulted in research on production of liquid fuels, such as butanol and ethanol, by fermentation. Butanol is a superior fuel [10] and has more calorific value than ethanol. In addition to fuel applications, butanol can be used in plastic manufacture and extraction of food flavors. It can be produced from renewable agricultural resources including cane molasses, agricultural biomass, corn, and dairy industry waste (whey permeate). The economics of butanol (and liquid fuels) has been studied extensively and the cost of substrate/raw material is one of the most influential factors impacting the economics of fermentation-derived liquid fuels [6,11,14–16]. For this reason, butanol should be produced from low-price substrates or waste agricultural residues such as soy molasses.

Soy molasses, a byproduct of the soy processing industry, contains a number of carbohydrates such as sucrose, dextrose, fructose, raffinose, pinitol, stachyose, and verbascose in addition to fat, flavonoids, protein, and minerals [4]. Currently, it is used as an inexpensive animal feed. Since soy molasses contains some fermentable carbohydrates, it can be used as an inexpensive fermentation substrate. During processing, the beans are defatted with hexane and then extracted with 60–70% aqueous ethanol. Ethanol is evaporated to give soy molasses containing approximately 10% solids. At this stage, it is a brown liquid with a pH of 5. Soy molasses is spray-dried and 2% tri-calcium phosphate is added as an anti-caking agent. This material is called spray-dried soy molasses (SDSM).

A few studies have examined the fermentation of soy molasses using lactic acid bacteria, which are capable of producing the enzyme α-galactosidase to hydrolyze pinitol, stachyose, verbascose, melibiose, and raffinose. Mital *et al.* [7] studied the fermentation of soymilk by lactic acid bacteria. These authors investigated the utilization of these sugars by *Lactobacillus plantarum* and *L. fermenti*. In another study, soy molasses was used as a substrate for the production of lactic acid by *L. salivarius* [8].

The objective of the present study was to produce butanol from SDSM using *Clostridium beijerinckii* BA101, a hyperbutanol-producing mutant strain. The composition of SDSM and the oligosaccharides present in soy molasses are shown in Table 1 and Figure 1, respectively.

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 at 80°C for 10 min and transferred aseptically to tryptone–glucose–yeast (TGY) extract medium [3]. A 5% (v/v) inoculum was used for these studies. Cells were grown anaerobically for 18–20 h at 34°C before they were transferred into solvent production medium containing sugars or SDSM as substrate.

Media preparation

Butanol was produced by *C. beijerinckii* BA101 when grown on glucose, fructose, sucrose, and galactose or SDSM solution. About 60–75 g l^{-1} sugar solution containing 1 g l^{-1} yeast extract (Difco Laboratories, Detroit, MI, USA) was sterilized at

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Table 1 Composition of SDSM

Component	g kg^{-1} (dry weight)
Dextrose	28.4
Sucrose	371.0
Fructose	34.2
Pinitol	29.5
Raffinose	46.1
Stachyose	178.0
Verbascose[a]	59.2
Protein	59.0
Fat[b]	80.0
Flavonoids	27.7
Moisture	30.4
Ash	57.4

[a,b]Amount may vary.

121°C for 15 min. On cooling to room temperature, the medium was transferred to an anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI) where it was kept for 24 h prior to inoculation with actively growing cells. Filter-sterilized P2 medium stock solutions [12] were added to the fermentation medium prior to inoculation. Tri-calcium phosphate or sodium chloride salt was added to the fermentation medium containing glucose at various concentrations prior to sterilization. These salts were obtained from Sigma Chemicals (St. Louis, MO).

SDSM was dissolved in distilled water to give a final concentration of 100 g l^{-1} total solids. This solution was stored at 4°C. Prior to sterilization, the SDSM solution was diluted to give desired total solids or fermentable sugar concentration and the pH was adjusted to 6.8 by adding 1 M NaOH. As stated above, yeast extract at 1 g l^{-1} was added to the medium. The solution was filter-sterilized through a 0.2-μm pore size polyethersulfone (PES) membrane filter unit (Nalgene, Rochester, NY). Diatomaceous earth (Sigma) was used as a filter aid. The SDSM medium was supplemented with P2 stock solution just before inoculation. The composition and the method of preparation of medium (P2) and stock solutions have been given elsewhere [12].

Fermentation

Batch fermentation studies were conducted in 50–100 ml screw-capped bottles containing either 60–75 g l^{-1} added or equivalent sugar concentration in P2 medium, which does not include non-metabolizable sugars. The bottles were inoculated with a TGY-grown culture of *C. beijerinckii* BA101 and incubated at 35°C for 120 h in a Coy anaerobic chamber with a modified atmosphere of 80% N_2, 15% CO_2, and 5% H_2. Samples were taken intermittently.

Analyses

The growth of *C. beijerinckii* BA101 was evaluated by measuring optical density at 540 nm. Fermentation samples were centrifuged in a microfuge centrifuge and the supernatant was stored at −18°C until analyzed for solvents and acid production. The cells were washed with 9 g l^{-1} sodium chloride solution and re-suspended in the same solution before measuring the optical density. This was done to remove medium ingredients, which may influence the optical density measurements. Removing the fermentation medium background color and ingredients is important to assure accurate optical density readings. The dry weight cell concentration was measured using a standard curve correlation between optical density and cell concentration.

Solvents (acetone, butanol, ethanol: ABE) and acids (acetic and butyric) were determined using a 6890 Hewlett-Packard gas chromatograph (Hewlett-Packard, Avondale, PA) equipped with a flame ionization detector (FID) and a 30 m×0.53 mm capillary column packed with FFAP (free fatty acid phase; polyethylene–glycol TPA-modified; Hewlett-Packard) [13]. The initial and final temperatures were 60°C and 160°C, respectively. Both injector and detector temperatures were 260°C. A 3390A Hewlett-Packard integrator was used to quantify the data. Nitrogen at 20 ml min^{-1} was the carrier gas. Samples (1 μl) were injected into the GC and propanol was used as an internal standard.

Carbohydrate utilization in pure sugar/SDSM solutions was determined using a Dionex BioLC HPLC (Dionex, Sunnyvale,

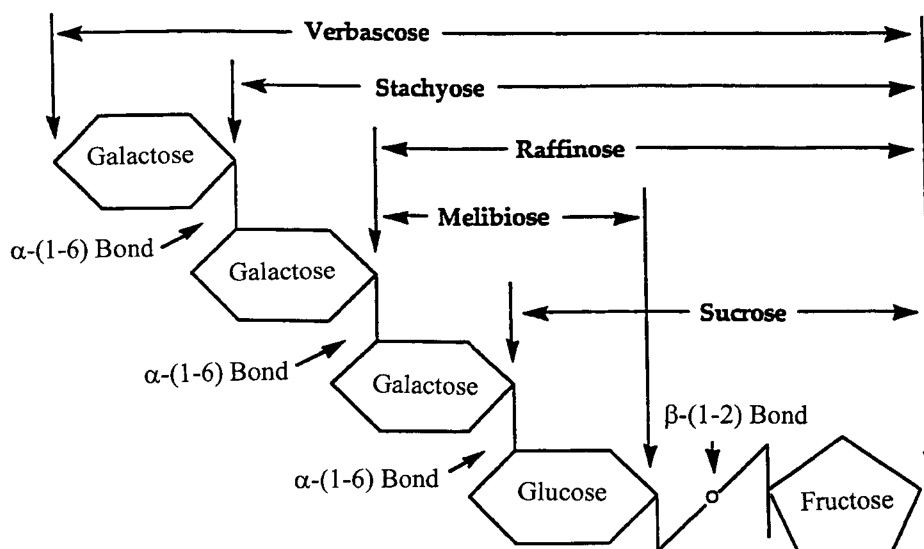


Figure 1 Predominant soy molasses oligosaccharides.

CA) fitted with a CarboPac PA-1 (4×250 mm) analytical column and a Carbopac PA-1 (4×250 mm) guard column. The system employed a Dionex pulsed electrochemical detector (PED). The PED was operated in the integrated amperometry mode. A Spectra-Physics AS 3500 autosampler (Spectra-Physics, San Jose, CA) was used. The mobile phase was 300 mM NaOH with a flow rate of 1 ml min^{-1}. A 50-μl sample was injected to the HPLC. The total run time for the sample was 45 min. Standards were prepared by diluting appropriate sugars in de-ionized water.

The specific growth rate constant (μ) and specific rate of solvent (ABE) production (ν) were calculated from time *versus* cell concentration and total ABE concentration, respectively. The specific growth rate constant $[(dX/dt)/X]$ is defined as the rate of cell growth per unit cell mass in the reactor at that time and is calculated by drawing a tangent on the log phase of the growth curve. Similarly, the specific rate of solvent production $[(dP/dt)/X]$ is defined as the rate of product formation per unit cell mass at that time and is calculated, similarly, from the ABE production *versus* time curve. In the above expression, X, P, and t represent cell concentration (g l^{-1}), solvent concentration (g l^{-1}), and time (h). The units for both specific growth rate constant and specific rate of product formation are h^{-1}. Both of these terms are indicative of fermentation conditions and their significance will be discussed in the *Results and Discussion* section.

The overall productivity (g l^{-1} h^{-1}) was calculated as the total solvent concentration achieved at the end of fermentation divided by the fermentation time, while the yield was calculated as the total solvents produced divided by the total sugar utilized and is expressed as g g^{-1}. In the overall productivity determination, acids were not included. Although the fermentations were run for up to 120 h, fermentation time was taken as the time when cells ceased to produce solvents, which usually was at 72–96 h.

Table 2 Fermentation parameters for ABE production using *C. beijerinckii* BA101

Control
C_s 23.8 [g l^{-1}], C_a 1.62 [g l^{-1}], C_{xm} 1.70 [g l^{-1}], T_L 6 h, T_f 72 h, C_{sb0} 60.1 [g l^{-1}], C_{sbf} 3.43 [g l^{-1}], $Y_{p/s}$ 0.42 [g g^{-1}], R_p 0.33 [g l^{-1} h^{-1}], ν 0.19 [h^{-1}], μ 0.06 [h^{-1}]

SDSM (80 g l^{-1})
C_s 10.7 [g l^{-1}], C_a 1.75 [g l^{-1}], C_{xm} 1.60 [g l^{-1}], T_L 8 h, T_f 96 h, C_{sb0} 34.7 [g l^{-1}], C_{sbf} 0.0 [g l^{-1}], $Y_{p/s}$ 0.31 [g g^{-1}], R_p 0.11 [g l^{-1} h^{-1}], ν 0.06 [h^{-1}], μ 0.05 [h^{-1}]

Tri-calcium phosphate (28.8 g l^{-1})
C_s 30.1 [g l^{-1}], C_a 1.56 [g l^{-1}], C_{xm} 1.65 [g l^{-1}], T_L 6 h, T_f 72 h, C_{sb0} 74.9 [g l^{-1}], C_{sbf} 4.9 [g l^{-1}], $Y_{p/s}$ 0.43 [g g^{-1}], R_p 0.42 [g l^{-1} h^{-1}], ν 0.35 [h^{-1}], μ 0.06 [h^{-1}]

*Sodium chloride (10 g l^{-1})**
C_s 21.5 [g l^{-1}], C_a 1.41 [g l^{-1}], C_{xm} 0.60 [g l^{-1}], T_L 9 h, T_f 96 h, C_{sb0} 62.4 [g l^{-1}], C_{sbf} 1.0 [g l^{-1}], $Y_{p/s}$ 0.35 [g g^{-1}], R_p 0.22 [g l^{-1} h^{-1}], ν 0.60 [h^{-1}], μ 0.05 [h^{-1}]

Nomenclature

C_a	Total acid concentration [g l^{-1}]
C_s	Total ABE concentration [g l^{-1}]
C_{sb0}	Initial sugar concentration [g l^{-1}]
C_{sbf}	Final sugar concentration [g l^{-1}]
C_{xm}	Maximum cell concentration [g l^{-1}]
R_p	Productivity [g l^{-1} h^{-1}]
T_L	Lag phase [h]
T_f	Fermentation time [h]
$Y_{p/s}$	ABE yield [g g^{-1}]
ν	Specific rate of ABE production [h^{-1}]
μ	Specific growth rate constant [h^{-1}]

*At higher concentrations, the culture resulted in longer lag phases and became acidogenic.

Results and discussion

Initially, a control batch fermentation was run using glucose as substrate and the products (ABE) obtained during the fermentation are shown in Figure 2 and Table 2. The culture produced 23.8 g l^{-1} total solvents in 72 h and the cells ceased to ferment at that time. Kinetic data on this fermentation are presented in Table 2. Similarly, batch fermentations were run using sucrose, fructose, galactose, raffinose, pinitol, verbascose, melibiose, or stachyose to evaluate if *C. beijerinckii* BA101 was able to utilize these sugars present in the soy molasses and produce solvents (ABE). *C. beijerinckii* BA101 utilized sucrose, fructose, and galactose in addition to glucose, but not raffinose, pinitol, melibiose, and stachyose. This is because this microorganism cannot hydrolyze the α1–6 glycosidic bond present in these sugars. The culture produced 23.4, 23.8, and 8.7 g l^{-1} total solvents from sucrose, fructose, and galactose, respectively, all at an initial concentration of 60 g l^{-1}.

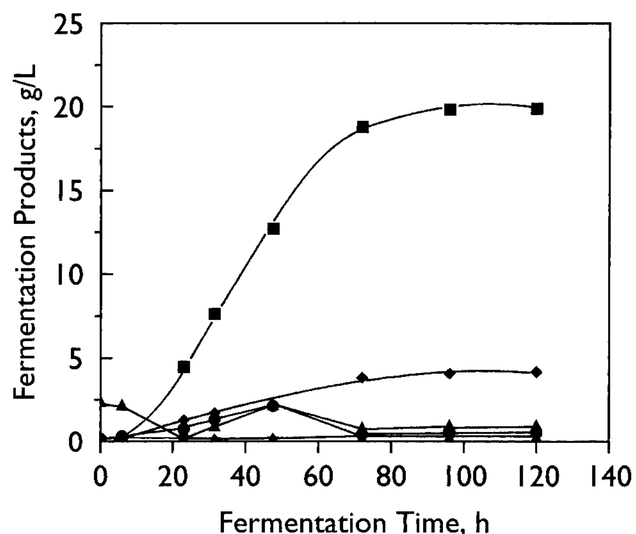


Figure 2 Production of ABE from glucose by *C. beijerinckii* BA101. (◆) Acetone. (■) Butanol. (★) Ethanol. (▲) Acetic acid. (●) Butyric acid.

Subsequently, a fermentation was carried out using 80 g l^{-1} SDSM (Figure 3A, Table 2). The total amount of solvent production using this substrate was 10.7 g l^{-1}. In addition to the solvents, *C. beijerinckii* BA101 also produced 1.6 g l^{-1} butyric acid. Compared to the glucose fermentation, *C. beijerinckii* BA101 produced low amounts of solvents from SDSM. All the fermentable sugars (dextrose, fructose, and sucrose) present in the medium were utilized. The reason for the limited solvent production was because of a deficiency of sugars in the fermentation medium containing 80 g l^{-1} SDSM. At time zero, there was 34.7 g l^{-1} total fermentable sugars in SDSM and their utilization by *C. beijerinckii* BA101 resulted in a solvent yield of 0.31 g g^{-1}.

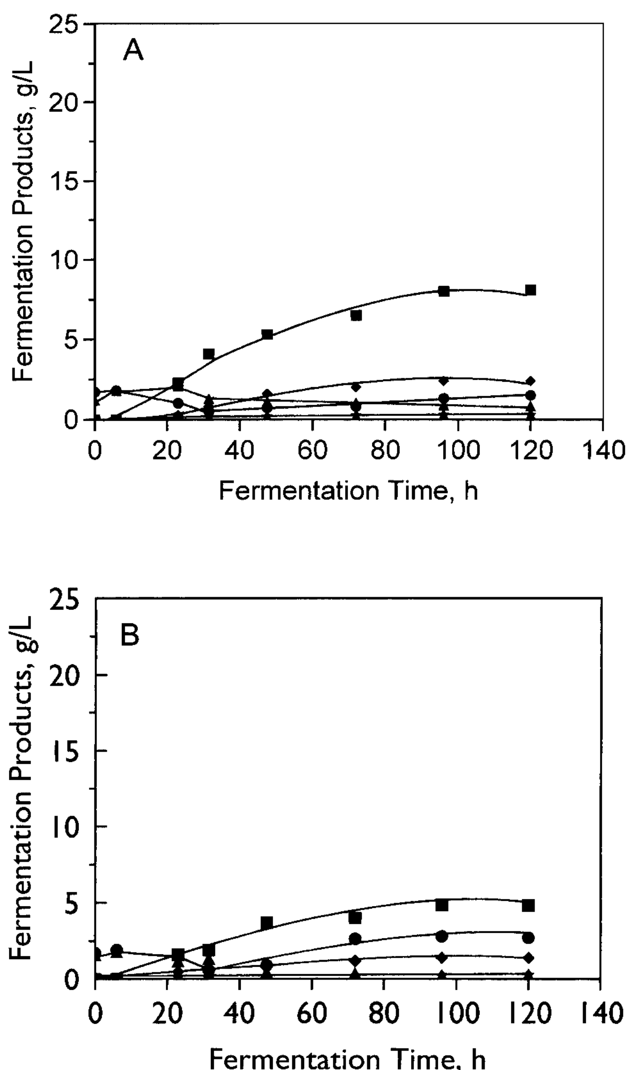


Figure 3 Production of ABE from SDSM using *C. beijerinckii* BA101. (A) 80 g l^{-1} initial SDSM. (B) 100 g l^{-1} initial SDSM. (◆) Acetone. (■) Butanol. (★) Ethanol. (▲) Acetic acid. (●) Butyric acid.

In order to increase the sugar concentration in the fermentation medium, the concentration of SDSM was increased to 100 g l^{-1}. This resulted in the production by *C. beijerinckii* BA101 of 6.3 g l^{-1} total solvents and 2.5 g l^{-1} butyric acid (Figure 3B). At the end of the fermentation, 22.4 g l^{-1} fermentable sugars remained unused. This led to speculation that inhibitory component(s) is(are) present in the fermentation medium, which interfere(s) with the fermentation of SDSM by *C. beijerinckii* BA101. In order to investigate this, 80 g l^{-1} SDSM was fermented with additional glucose (25.3 g l^{-1}). Total solvents accumulated to 22.8 g l^{-1} (Figure 4) and there was 0.8 g l^{-1} unused fermentable sugar in the broth. Thus, SDSM contains component(s) that inhibits the fermentation.

Like corn steep liquor (CSL), a byproduct of the corn wet milling process, soy molasses is a byproduct of the soybean-processing industry. While CSL is a rich source of growth promoters, it also contains growth inhibitors such as lactic acid, phytic acid, heavy metals (Zn, Cu), minerals (Na, K, S, P, Mg), and their salts [2], which must be diluted several times to reduce their inhibitory effect before CSL can be used as nutrient source for fermentation [9]. Similarly, soy molasses may contain high concentrations of inhibitors.

Mineral salts are inhibitory to *C. acetobutylicum* P262 [5]. At a 30 g l^{-1} sodium chloride concentration, no growth was reported. Table 1 shows that 80 g l^{-1} SDSM contained 4.6 g l^{-1} minerals (calculated). The total salt concentration of their respective salts in the fermentation medium containing 80 g l^{-1} SDSM would be much higher than the mineral content. Tri-calcium phosphate is added to liquid soy molasses as an anti-caking agent prior to spray-drying. In addition to 2% tri-calcium phosphate, SDSM also contains other salts.

To examine the inhibitory effect of salts on the ABE fermentation by *C. beijerinckii* BA101, tri-calcium phosphate and sodium chloride were chosen. Fermentations were run with various amounts of tri-calcium phosphate and the results suggest that this salt was not inhibitory to the fermentation when present up to a concentration of 43.1 g l^{-1} (Figure 5A). Instead, calcium phosphate was beneficial to the fermentation as the total amount of solvents increased from 23.8 to 30.1 g l^{-1} (ABE ratio 6:30:1). The total acid concentration did not increase with increased amounts of added calcium phosphate. An enhancing affect on butanol production was observed when sodium acetate was added to cultures of *C. beijerinckii* NCIMB 8052/*C. beijerinckii* BA101 [1]. The effect of tri-calcium phosphate may have been similar to that of sodium acetate.

Experiments were conducted with various amounts of sodium chloride added to the fermentation medium. The results are presented in Figure 5B. The total amount of solvents decreased while acids increased with increased amounts of sodium chloride. At a sodium chloride concentration of 20 g l^{-1}, the lag phase for cell growth was 20 h while at 30 g l^{-1}, it was over 80 h.

The specific growth rate (μ) and specific rate of product formation (ν) are plotted in Figure 6A as a function of the tri-calcium phosphate concentration. At 0 g l^{-1} added tri-calcium phosphate, ν was 0.19 h^{-1} and at 28.8 g l^{-1} added calcium phosphate, ν was 0.35 h^{-1}, suggesting that *C. beijerinckii*

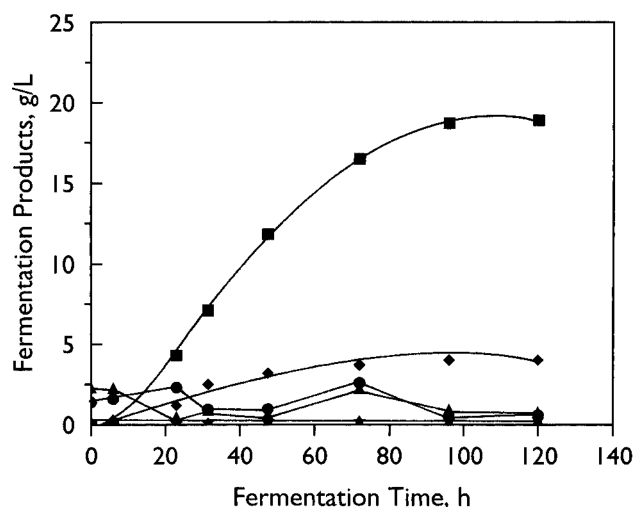
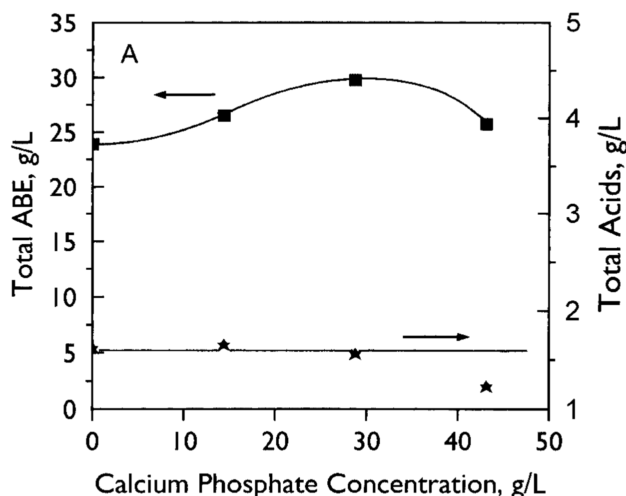


Figure 4 Production of ABE from 80 g l^{-1} SDSM supplemented with 25.3 g l^{-1} glucose using *C. beijerinckii* BA101. (◆) Acetone. (■) Butanol. (★) Ethanol. (▲) Acetic acid. (●) Butyric acid.

BA101 produced more solvents per gram of cells in the presence of an elevated concentration of tri-calcium phosphate. While ν values increased with increased concentration of tri-calcium phosphate, μ remained constant at 0.06 h^{-1}. These values of μ and ν were calculated from exponential growth/ABE production phase data.

Similarly, the values of μ and ν were plotted against various concentrations of sodium chloride (Figure 6B). With increased amounts of sodium chloride, μ decreased linearly. At a sodium chloride concentration of 10 g l^{-1}, ν increased significantly, suggesting that more solvent was produced per gram of cells (21.5 g l^{-1}; Figure 5B). This concentration of sodium chloride may represent an optimal concentration for the microorganism for solvent production. At 10 g l^{-1} sodium chloride concentration, a maximum cell concentration of 0.6 g l^{-1} was achieved in the fermentation broth (Table 2), while in the control experiment, it was 1.7 g l^{-1}.

These studies suggest that SDSM can be a useful substrate for producing solvent if it is used at or less than 80 g l^{-1} and



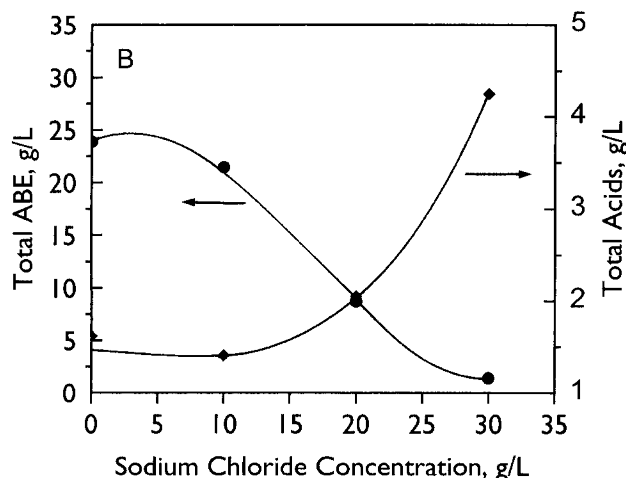
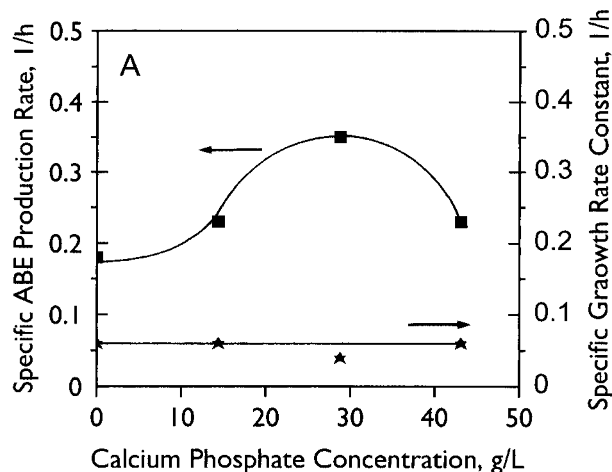


Figure 5 Production of total solvents and acids supplemented with salts. (A) Tri-calcium phosphate: (■) total ABE; (★) total acids. (B) Sodium chloride: (●) total ABE; (◆) total acids.



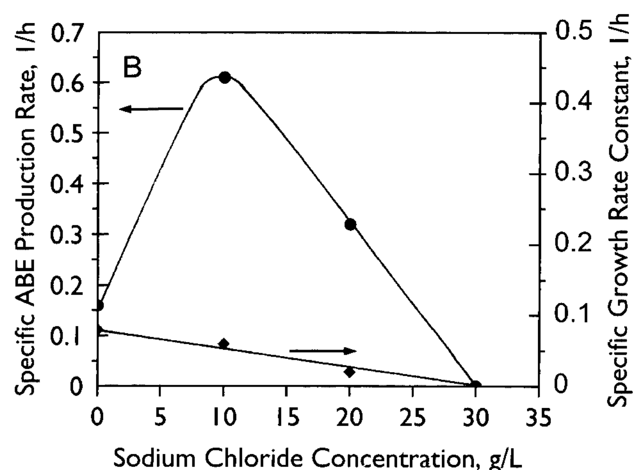


Figure 6 Specific growth rates and specific solvent production rates from glucose in the presence of salts for *C. beijerinckii* BA101. (A) Tri-calcium phosphate: (■, ν) total ABE; (★, μ). (B) Sodium chloride: (●, ν) total ABE; (◆, μ).

additional sugar, such as glucose or sucrose, is added to the fermentation medium. In conclusion, *C. beijerinckii* BA101 can ferment glucose, sucrose, fructose, galactose, and maltodextrin [3]. The culture is not inhibited by tri-calcium phosphate at concentrations up to 43.1 g l^{-1}, while sodium chloride inhibits the culture at concentration over 10 g l^{-1}. Tri-calcium phosphate (28.8 g l^{-1}) enhanced production of ABE from 23.8 to 30.1 g l^{-1}. *C. beijerinckii* BA101 is unable to ferment raffinose, pinitol, verbascose, and stachyose. The total amount of these non-fermentable sugars in SDSM is 313 g kg^{-1} (dry weight). Genetic manipulation of *C. beijerinckii* BA101 offers an approach to produce a strain, which is able to produce α1–6 glycosidase enzymes. Once this bond is cleaved, the culture would be able to utilize raffinose, pinitol, verbascose, melibiose, and stachyose to produce ABE.

Acknowledgements

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