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Acetone-Butanol-Ethanol (ABE) Production in Fermentation of Enzymatically Hydrolyzed Cassava Flour by *Clostridium beijerinckii* BA101 and Solvent Separation

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Cassava constitutes an abundant substrate in tropical regions. The production of butanol in ABE fermentation by *Clostridium beijerinckii* BA101 using cassava flour (CF) was scaled-up to bioreactor level (5 L). Optimized fermentation conditions were applied; that is, 40°C, 60 g/l CF, and enzymatic pretreatment of the substrate. The batch fermentation profile presented an acidogenic phase for the first 24 h and a solventogenic phase afterwards. An average of 37.01 g/l ABE was produced after 83 h, with a productivity of 0.446 g/l/h. Butanol production was 25.71 g/l with a productivity of 0.310 g/l/h, high or similar to analogous batch processes described for other substrates. Solvent separation by different combinations of fractioned and azeotropic distillation and liquid-liquid separation were assessed to evaluate energetic and economic costs in downstream processing. Results suggest that the use of cassava as a substrate in ABE fermentation could be a cost-effective way of producing butanol in tropical regions.

Keywords: Butanol, ABE fermentation, cassava, *Clostridium beijerinckii*, hydrolysis

Introduction

Nowadays, energy required for the chemical industry is based mainly in fuels such as petroleum and coal; nevertheless, owing to economic and strategic considerations, research is focused on new alternatives for the synthesis of chemical substances. Fermentative production of solvents derived from cellulose wastes and prime material with high concentrations of starch or glucose may provide significant economical and technological benefits.

One of the processes of great biotechnological interest is acetobutylic fermentation, where the products obtained include butanol, acetone, and ethanol, and therefore it is also known as ABE fermentation. Although some decades ago, the butanol produced by fermentation could not compete with petrochemically derived butanol, it has attracted increasing interest because of the fluctuation of global petroleum prices and the uncertainty of future raw oil supply. As a biofuel, butanol is more valuable than ethanol, as it has desirable properties, which include higher energy content, higher boiling point, and a reduced need to modify combustion energies; in addition, it has applications in food and plastic industries, among others [26]. The ABE fermentation process may be carried out by bacteria of the genus *Clostridium*. Several butanol-producing strains have been reported, including *Clostridium acetobutylicum* P262 (renamed as *C. saccharobutylicum*), *C. acetobutylicum* ATCC 824, *C. acetobutylicum* NRRL B643, *C. acetobutylicum* B18, *C. beijerinckii* P260, *C. beijerinckii* BA101, *C. beijerinckii* LMD 27.6, *C. butylicum*, *C. aurantibutyricum*, and *C. tetanomorphum* [16].

The economics of butanol production by fermentation is affected by several factors; one of the most important is the cost of the substrate, and thus the availability of

inexpensive raw materials is necessary to develop an economically feasible process. To date, butanol production has been evaluated with multiple substrates such as maltodextrin, wheat straw hydrolysate, corn-derived waste, packing peanuts, and soy molasses [4, 9, 17, 18, 22]. However, other potential substrates, especially from tropical regions, have been overlooked.

In this respect, the most abundantly available, cost-effective raw materials used for the fermentation industry are starchy materials [25]. Cassava (*Manihot esculenta*) is an important source of food and calories for the population of tropical countries in Asia, Africa, and Latin America [13], where it is basic food for more than 700 million people, thus ranking as the world's sixth most important food crop [24]. World production reached 162 million ton in 1998, with Africa being the largest producer (53%), followed by Asia (29%) and Latin America (18%). About 60% of the cassava produced is employed for human consumption, whereas 33% is used in the animal food industry and only 7% by industries, including textile, paper, and fermentation [13]. Despite its small size, Costa Rica has an important production of cassava, with crops yielding between 60 and 98 ton/ha. The remarkable capacity of cassava to adapt to different agro-ecological conditions and its non-requirement of specific growth conditions permit the cultivation of cassava all year round in Costa Rican territory and potentially in any lowland, although current production takes place mostly in the northern and Atlantic regions. Therefore, and considering its high starch content (20%–25%, wet basis), cassava is a promising crop for biofuel production from renewable resources. Interest in the use of cassava to produce butanol is quite recent, with only a few reports in the last 4 years [6, 11, 12, 25, 26].

One of the most significant challenges faced by commercial butanol fermentation was the prohibitive cost of the recovery of butanol from broth, due both to its low concentration and higher boiling point than water [20]. At such low concentration of solvents, the energy required for butanol separation by traditional distillation is higher than the energy content of the product itself [5]. Product-removal techniques, including gas stripping, adsorption, liquid-liquid extraction, pervaporation, and reverse osmosis, have been described [16]. However, the downstream process is still a challenge to obtain a sustainable process.

This paper aimed to describe the butanol production from cassava flour (CF) fermentation at a laboratory bioreactor scale, with *Clostridium beijerincki* BA101, a butanol-hyperproducing strain generated by chemical mutagenesis [1]. Although this strain has been used with several substrates, only one previous study employs cassava [12], but at a flask scale. This is the first report of ABE production with cassava and *C. beijerinckii* BA101 at a reactor scale. The efficiency of butanol recovery by means of several combinations of conventional methods is also presented.

Materials and Methods

Bacterial Strain and Inoculum Preparation

The hyperbutanol-producing strain *C. beijerinckii* BA101, acquired from the American Type Culture Collection (ATCC), code ATCC PTA-1550, was employed in the fermentation studies. Aliquots of 0.1 ml of spore solution were transferred to tubes containing thioglycolate broth (Scharlau, Barcelona, Spain). Inoculums were prepared by heat shocking the tubes for 5 min at 80°C and incubating under anaerobic conditions for 48 h at 42°C.

CF Production

Cassava roots (Mangi variety) were obtained from La Garita, Alajuela, Costa Rica. CF was produced as described by Lépiz-Aguilar *et al*. [11].

CF Hydrolysis

CF was hydrolyzed by means of enzymatic treatment. Adequate amounts of CF were soaked in distilled water to obtain a suspension of 60 g/l CF. Enzymatic hydrolysis consisted of two steps: liquefaction and saccharification. Prior to liquefaction, CF suspensions were supplemented with $CaCl_2$ (1 g/l) to obtain a concentration of 40 mg-Ca/l and the pH was adjusted to 6.5 with 1 M NaOH. For liquefaction, flasks were placed in a bath shaker (150 rpm) at 93°C, and α-amylase (Termamyl, 120 KNU/g; Novozymes Corp., Denmark) was added at 1 ml/kg starch. The enzymatic reaction was carried out for 2 h, followed by cooling in a water bath and a decrease in pH to 4.5 with 1 M HCl. Next, saccharification was performed by adding β-glucoamylase (amyloglucosidase, 300 AGU/ml; Novozymes Corp.) at 1.7 ml/kg starch and mixing in a bath shaker (150 rpm) for 6 h at 60°C and 34 h at 40°C. After saccharification, the enzyme was inactivated by heating for 5 min in a bath at 80°C.

Bioreactor and Fermentation Conditions

Fermentations were conducted in batch mode in a 5 L bioreactor (Electrolab Inc., USA) containing an effective volume of 4 L and 60 g/l hydrolyzed CF. The filled reactor was autoclaved at 121°C for 20 min. Upon cooling at room temperature and under oxygen-free nitrogen gas conditions (flux ~69 kPa), filter-sterilized P2 medium stock solutions (buffer: KH_2PO_4, 50 g/l; K_2HPO_4, 50 g/l; ammonium acetate, 220 g/l; vitamin: *para*-aminobenzoic acid, 0.1 g/l; thiamin, 0.1 g/l; biotin, 0.001 g/l; mineral: $MgSO_4{\cdot}7H_2O$, 20 g/l; $MnSO_4H_2O$, 1 g/l; $FeSO_4{\cdot}7H_2O$, 1 g/l; NaCl, 1 g/l [9]) were added (10 ml each per 970 ml of hydrolyzed CF solution). In order to remove the air by atmosphere-displacing, a N_2 flux (~69 kPa)

was applied for 30 min over the medium prior to inoculation. Batch cultures were initiated by adding 5% (v/v) inoculum [3, 14], prepared as previously described. Fermentations were run for 96 h at 40°C, and continuous homogenization was achieved by mechanical agitation at 150 rpm. A continuous flow of N_2 was supplied. The pH was not controlled in fermentations, but it was initially adjusted to 4.5. Samples from the fermentation mixture were periodically withdrawn, centrifuged at 3,000 rpm for 30 min, and the supernatant was filtered (nitrocellulose membranes, 0.22 μm pore diameter) for analytical determinations. Raw samples were employed to monitor the bacterial population.

Solvent Separation Studies

Butanol separation by classical methods was performed from the filtrated final mixture in the fermentation broth. Different separation processes, including the techniques of fractional distillation (FD), azeotropic distillation (AD), and liquid-liquid extraction (LLE), were considered (Fig. 1). Within FD, direct separation (the light component is initially separated) and indirect separation (the heavy component is initially separated) were applied to obtain the mixture of butanol/water or the butanol/water azeotrope (separation plans A, SP_A), which were further separated by combinations of LLE with ethyl ether and distillation (separation plans B, SP_B). Fig. 1 details the separation strategies employed. Seven separation methods (SMs), the result of combinations of SP_A and SP_B (detailed in Table 2), were performed in order to determine the energetic consumption as an estimation of costs. The purity of the components was determined after separation.

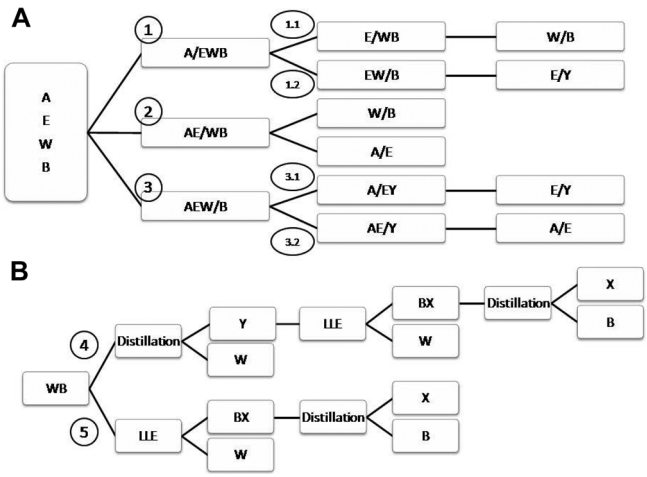

Fig. 1. Methods of butanol separation from the fermentation broth.

(**A**) Separation plans (SP_A): SP_A1.1, sequential direct separation of light components to obtain the mixture BW; SP_A1.2, combined direct-indirect separation to obtain Y; SP_A2, separation in two binary-mixtures with further direct separation to obtain the mixture BW; SP_A3.1, combined indirect-direct separation to obtaining Y; SP_A3.2, sequential indirect separation to obtain Y. (**B**) Separation plans of mixtures butanol/water (SP_B), obtained in SP_A methods: SP_B1, separation of B (azeotropic composition) from W by AD-LLE-FD sequence; SP_B2, separation of B by LLE-FD sequence. A: acetone; B: butanol; E: ethanol; W: water; X: ethyl ether; Y: butanol/water azeotrope; LLE: liquid-liquid extraction.

Analytical Methods

ABE was measured using a 5890 Hewlett Packard gas chromatograph (Hewlett Packard, USA) equipped with a flame ionization detector (FID) and a 30 m × 0.25 mm column (Supelco SPB-5, USA). Glucose and total reducing sugar concentrations were determined according to the glucose oxidase enzymatic Trinder method and the Nelson-Somogyi method, respectively [23]. Productivity was calculated as the ABE concentration achieved (g/l) divided by the fermentation time. Cell population was determined as colony forming units per milliliter (CFU/ml) by the plate count technique on SPS (Difco, BD, USA) agar plates. Plates were incubated for 48 h at 42°C in anaerobic jars (HP11; Oxoid, UK), and anaerobic conditions were created with AnaeroGen AN35 envelopes (Oxoid).

Results and Discussion

ABE Production in Batch Fermentation of CF

The use of cassava flour as fermentation substrate was assessed for batch ABE production at reactor scale. Scarce reports deal with the application of cassava [6] and only one employed the butanol-hyperproducing strain *C. beijerinckii* BA101 [11]. Moreover, in those studies, the production was performed at flask scale. The work by Lépiz-Aguilar *et al.* [11] defined optimal operation conditions for butanol production from cassava and this strain, which were used in the present study: working temperature of 40°C, 60 g/l CF, and an enzymatic pretreatment of the substrate. The use of 40°C differs from most ABE processes with *C. beijerinckii* BA101, which have been performed at temperatures varying from 30°C to 35°C [2–4, 9, 14, 15]. The use of chemical pretreatment of the substrates usually derives in the production of fermentation inhibitors such as salts, phenolic acids, and aldehydes [3, 19, 21]. Therefore, enzymatic pretreatments are the best option in order to avoid the removal of inhibitors. Although *C. beijerinckii* is a hyperamylase-producing microorganism, poor solvent production was obtained without pretreatment of the CF (data not shown).

The runs were performed for 96 h. In the first hours of fermentation a slight increase in the concentration of reducing sugars was observed (Fig. 2), which could be due to a residual saccharification effect of the enzymatic

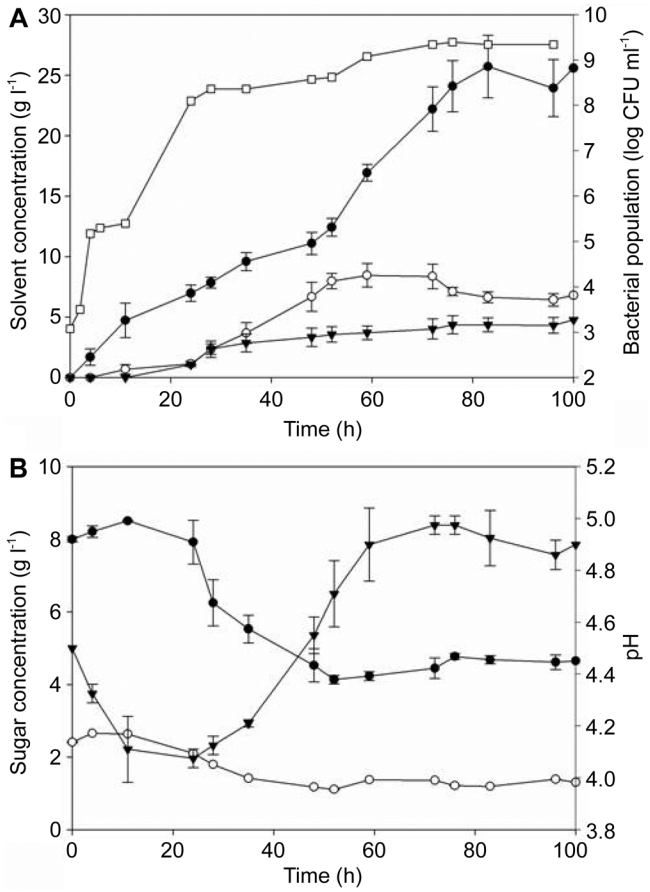

Fig. 2. ABE production and growth profile of *C. beijerinckii* BA101 grown on cassava flour.

(**A**) Bacterial growth and production of ABE from CF (60 g/l) fermentation by *C. beijerinckii* BA101. (□) Bacterial population; (●) butanol; (○) ethanol; (▼) acetone. (**B**) Reducing sugars (●), glucose (○), and pH (▼) profiles during the process. Values plotted are means ± standard deviation for duplicate batch reactor fermentations (except for bacterial population in **A**).

pretreatment. The pH in the system varied between 4 and 5 (Fig. 2); the acidogenic phase lasted for the first 24 h, where production of acetate and butyrate are favored. In this period, however, nearly 25% of the total butanol was produced, and a pseudo lag-phase in bacterial growth was observed between 4 and 11 h, coincident with the pH drop. At this point, a metabolic shift to solvent production (solventogenic phase) was observed, that eventually led to the increase in pH due to the uptake of the acid compounds [10].

The process yielded a total solvent production of 37.01 g/l, corresponding to a productivity of 0.386 g l^{-1} h^{-1}. However, the maximum butanol concentration was obtained after 83 h and remained almost constant until the end of the

Table 1. Kinetic parameters in the performance of 5L batch reactors for ABE production from CF by *C. beijerinckii* BA101.

Parameter	Mean value
Fermentation time (h)	96
Total ABE, Cn_{ABE} (g/l)	37.01
ABE productivity 96 h, P_{ABE} (g/l/h)	0.386
ABE productivity 83 h, P_{ABE} (g/l/h)	0.446
Product ratio, B:E:A (Adim)	1.0:5.6:1.5
Time of maximum butanol concentration, t_{Bmax} (h)	83
Maximum butanol concentration, Cn_{Bmax} (g/l)	25.71
Butanol productivity 96 h, P_B (g/l/h)	0.268
Butanol productivity 83 h, P_B (g/l/h)	0.310
Time of maximum ethanol concentration, t_{Emax} (h)	58
Maximum ethanol concentration, Cn_{Emax} (g/l)	6.93
Ethanol productivity 96 h, P_E (g/l/h)	0.072
Ethanol productivity 83 h, P_E (g/l/h)	0.053
Time of maximum ethanol concentration, t_{Amax} (h)	79
Maximum acetone concentration, Cn_{Amax} (g/l)	4.37
Acetone productivity 96 h, P_A (g/l/h)	0.046
Acetone productivity 83 h, P_A (g/l/h)	0.080

fermentation (Fig. 2); therefore, an 83 h productivity is a more accurate indicator of the efficiency of the process, and corresponds to 0.446 g l^{-1} h^{-1}. By this time, butanol yield reached 25.71 g/l and a productivity of 0.310 g l^{-1} h^{-1}, high compared with other batch systems [22], and similar to that obtained with straw by Qureshi *et al*. [21]. This scale-up resulted in slightly higher ABE levels compared with the experiments in flasks performed at the same conditions (total solvent concentrations from 32 to 35 g/l [11]). In addition, the results are promising, as concentrations of around 20–25 g/l are usually considered as inhibitory for the fermentation process [7, 8]. Gu *et al*. [6] reported productivities of 0.165 and 0.257 g l^{-1} h^{-1} for butanol and total solvents, respectively, using CF and *C. acetobutylicum*. In an interesting approach, Tran *et al*. [26] described the ABE production from cassava starch by means of using *Bacillus subtilis* in a co-culture with *Clostridium butylicum*, in order to enhance the reduction of the medium (redox potential) and to take advantage of the amylolytic potential of *B. subtilis*. However, the maximum production obtained was only 9.71 g/l ABE (6.7 g/l butanol), much lower than the one reported in the present work. Similarly, Thang *et al*. [25] reported an ABE production of 21.0 and 19.4 g/l (16.9 and 15.5 g/l) from cassava starch and cassava chips, respectively, by *Clostridium saccharoperbutylacetonicum* N1-4,

with productivity values of 0.44 and 0.40 g l^{-1} h^{-1}. Although the productivity reported in the present work was in the same range, our absolute production was more than 75% higher.

Other works of ABE fermentation in batch systems with *C. beijerinckii* BA101 report total solvent production values ranging from 8.6 to 21.7 g/l for diverse substrates such as soy molasses, corn fiber hydrolysate, packing peanuts,

Table 2. Separation of butanol by different methods and estimation of energy and economic costs.

Separation method	Sequence of separation (according to Fig. 1)	Phase separation phase 1/ phase 2 (purity of the fraction)	Energy consumed (kJ/l)	Cost (USD/100 L)
SM1	$SP_A1.1$-SP_B1	A (99)/EWB E(93)/WB Y(B 41)/W(99) XB/W(99) X(99)/B(99)	720.3	7.0
SM2	$SP_A1.1$-SP_B2	A(99)/EWB E(93)/WB XB/W(94) X(99)/B(99)	197.4	2.0
SM3	$SP_A1.2$-SP_B1	A(99)/EWB EW/B(W 98) E(94)/Y(B 40) XB/W(99) X(99)/B(99)	974.4	9.6
SM4	SP_A2-SP_B1	AE/WB A(99)/E(94) Y(B 42)/W(99) XB/W(99) X(99)/B(99)	799.1	7.8
SM5	SP_A2-SP_B2	AE/WB A(99)/E(94) XB/W(92) X(99)/B(99)	276.2	2.8
SM6	$SP_A3.1$-SP_B1	AEW/B(W 99) A(98)/EY E(93)/Y(B 41) XB/W(98) X(99)/B(99)	839.0	8.2
SM7	$SP_A3.2$-SP_B2	AEW/B(W 99) AE/Y(B 40) A(98)/E(93) XB/W(98) X(99)/B(99)	920.9	9.0

wheat straw, and agricultural wastes [9, 15, 17, 18]. Other parameters from the fermentations are presented in Table 1. As far as the authors know, this is the first report of ABE production at a bioreactor scale by *C. beijerinckii* using CF as the fermentation substrate.

Solvent Separation Strategies

The final fermentation broth was subjected to different separation processes in order to estimate the associated energy cost. The separation scheme included unitary operations such as FD, AD, and LLE. The total energy and economic costs related to the strategies are shown in Table 2. Strategies SM2 and SM5, in which direct liquid-liquid separation was applied to the butanol-water mixture, showed the lowest energy requirements (SP_B2) and consequently the lowest cost. The methods that involved the separation of butanol-water through SP_B1 obtained the mixture in its azeotropic composition, thus leading to a high energy demand. However, the application of LLE showed a reduction in efficiency when applied to methods that included the plan SP_B2 in respect to those that used SP_B1, in terms of recovery; that is, in SP_B1, the average concentration of butanol in the refined fraction was 1.1%, as opposed to 6.5% in SP_B2 (see purity of the refined fraction "W" in Table 2). The data indicate that application of LLE to the butanol-water mixture with an excess of water (and not with the mixture at azeotropic concentration) results in an increased loss of butanol due to the partial solubility of butanol in water.

Therefore, and considering that high butanol concentrations are expected in the fermentation process, a separation method that maximizes the recovery should be employed. Consequently, from a technical point of view, the methods including the SP_B2 plan are not recommended, despite being the most economic. Putting it all together, the most recommended method is SM1, as it is fairly cost-effective and allows good purity of the target compounds. This exercise did not intend to make an exhaustive economic analysis of the cost-effectiveness of the process, but only an estimation in terms of cost related to separation by traditional means. A complete economical analysis should be conducted to define the suitability of the process.

Alternative recent technologies for butanol downstream processing from ABE fermentation should be adapted and evaluated in the proposed process with CF. In this context, gas stripping has been a popular butanol recovery method, as it presents features such as relatively simple operation, safety to the culture, low energy input, and reduced facility costs. Moreover, Xue *et al*. [27] proposed a two-stage gas stripping for butanol recovery from ABE fermentation with *C. acetobutylicum* JB200 in a fibrous bed bioreactor, which was effective in producing high-titer butanol, easily purified with less energy consumption.

The study has shown the feasibility of scaling-up ABE production from CF fermentation by the high butanol-producing strain *C. beijerinckii* BA101, to bioreactor level and with enzymatic pretreatment of the substrate. Production parameters were comparable or better to those obtained with other well-studied substrates in batch operation, and maintained the productivity levels previously described at flask scale. Separation of butanol from the fermentation broth by direct FD to obtain a water-butanol mixture and subsequent separation of butanol (azeotropic composition) from the water by an AD-LLE-FD sequence seemed to be the most recommended downstream processing method.

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