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SHORT COMMUNICATION

Butanol production by *Clostridium beijerinckii* BA101 using cassava flour as fermentation substrate: enzymatic versus chemical pretreatments

Leonardo Lépiz-Aguilar · Carlos E. Rodríguez-Rodríguez · María Laura Arias · Giselle Lutz · William Ulate

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Abstract Cassava flour (CF), a cost-effective source of starch, was employed as a substrate for successful acetone-butanol-ethanol (ABE) production by batch-fermentation with *Clostridium beijerinckii*. The effect of temperature, initial concentration of CF and chemical/enzymatic hydrolysis were studied in a 2^3 factorial design. Results revealed that temperature and initial concentration of substrate exert a significant effect on ABE production, as well as interactions of temperature with the other variables. Solvent production was maximized when working at 40°C, 60 g l^{-1} CF and enzymatic pretreatment. An average of 31.38 g l^{-1} ABE was produced after 96 h, with a productivity of 0.33 g l^{-1} h^{-1}. A posterior randomized block design (3 × 2) showed that enzymatic hydrolysis (with saccharification periods of 6 h at 60°C) enhances both reducing sugar and solvent production if compared to chemical pretreatments. Average ABE production in this case was 27.28 g l^{-1}, with a productivity of 0.28 g l^{-1} h^{-1}. Results suggest that CF may be a suitable substrate for industrial ABE production.

Keywords Cassava · Butanol · ABE fermentation · Hydrolysis · *Clostridium beijerinckii*

L. Lépiz-Aguilar · W. Ulate
School of Chemical Engineering, Universidad de Costa Rica (UCR), 2060 San José, Costa Rica

C. E. Rodríguez-Rodríguez · M. L. Arias
Food and Water Microbiology Laboratory, Faculty of Microbiology, UCR, 2060 San José, Costa Rica

G. Lutz
School of Chemistry, UCR, 2060 San José, Costa Rica

W. Ulate
Costa Rican Petroleum Refinery (RECOPE), 4351-1000 San José, Costa Rica

Present Address:
C. E. Rodríguez-Rodríguez (✉)
Unitat de Biocatàlisi Aplicada associada al IQAC (CSIC-UAB) and Departament d'Enginyeria Química, Escola d'Enginyeria, Universitat Autònoma de Barcelona, 08193 Bellaterra, Spain
e-mail: carlos.rodriguezrodriguez@ucr.ac.cr

Introduction

As a result of increasing oil prices, constant conflicts in the oil-supply regions and depletion of fossil fuels, research around the world has focused on the production of fuels such as ethanol and butanol, obtained from renewable resources (Qureshi et al. 2007, 2008). Butanol has developed an important worldwide market, reaching to 2.9 billion pounds in the United States by 2007 (Ezeji et al. 2007). Historically, although butanol was employed during WW I and WW II, its production by fermentation could not compete with petrochemical industry and the last biobutanol plant ceased operations in the early 1980s in South Africa (Qureshi and Blaschek 2001a; Qureshi et al. 2007). However, due to recent advances in biotechnology and bioprocessing, commercial interest has returned to butanol fermentation and current research has been directed towards the development of better processes and microbial hyperproducing-strains.

Butanol can be produced by different microorganisms, including *Clostridium acetobutylicum* (Madihah et al. 2001) and *C. beijerinckii* (Formanek et al. 1997), through the fermentation of several substrates, by the so called ABE fermentation, which also produces acetone and ethanol (Qureshi et al. 2006). It has been demonstrated that the fermentation substrate is one the most important factors

that influences the price of butanol (Qureshi and Blaschek 2000), therefore, production should be focused towards the use of low price substrates or agricultural residues (Qureshi et al. 2001). Studies in the last 15 years have demonstrated butanol production from multiple substrates (Formanek et al. 1997; Parekh et al. 1998, 1999; Jesse et al. 2002; Qureshi et al. 2006, 2007); nonetheless, abundant substrates widely available in tropical latitudes such as Costa Rican territory have been barely explored. Costa Rica produces an abundant supply of cassava; this is the root from the plant *Manihot esculenta*, with an average starch content of approx. 20–25% in wet basis. In Costa Rica crops yield between 60 and 98 ton ha^{-1}, and though most of the production is carried out in the northern and Atlantic regions, any lowland is suitable for cassava cultivation all year round. Therefore, considering its high production, proper conditions for cultivation and nutritional features (high starch content), cassava may be an interesting substrate for ABE fermentation processes in tropical regions.

The objectives of the present study included the evaluation and optimization of the conditions for butanol production by fermentation of cassava flour (CF), using *C. beijerinckii* BA101. This strain is a butanol-hyperproducing, hyperamylolytic mutant that was generated by chemical mutagenesis (Annous and Blaschek 1991) and features a series of advantages related to solvent production in ABE fermentation (Qureshi and Blaschek 2001b).

Materials and methods

Bacterial strain and inoculum preparation

A hyperbutanol producing strain of *C. beijerinkii* BA101 (ATCC 35702) was employed in the 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. The roots were manually peeled and cut into small pieces; visually rotten parts were removed as well as the inner fiber. Cassava was dried in a drying tunnel until a humidity content of ~9–12%. The pieces were ground to fine particles in a hammermill and the resultant flour was sifted (mesh #100, 150 μm particle size), packed in polypropylene bags and stored at environmental conditions until use. The chemical composition of CF was determined by the Research Center of Food Technology (CITA) according to national (AQCITA: M002, M004, M003, M009, M018) or international (AOAC: 925.09, 923.03, 979.09) standards.

CF pretreatment and hydrolysis

CF was hydrolyzed by HCl treatment or enzyme hydrolysis. Adequate amounts of CF were soaked in distilled water to obtain suspensions of 60, 80 and 90 g l^{-1} CF. For the acid chemical treatment (CT), a solution of HCl 1 M was added to a final pH of 1.5. The flasks were then autoclaved at 121°C for 2 h, cooled with tap water to stop hydrolysis reaction and kept at 4°C until use. Enzymatic hydrolysis consisted of two steps: liquefaction and saccharification. Prior liquefaction, CF suspensions were supplemented with $CaCl_2$ (1 g l^{-1}) to obtain a concentration of 40 mg Ca l^{-1} and pH was adjusted at 6.5 with NaOH 1 M. For liquefaction, flasks were placed in a bath shaker (150 rpm) at 93°C and α-amylase (Termamyl, 120 KNU g^{-1}, Novozymes Corp., Bagsvaerd, Denmark) was added at 1 ml kg^{-1} starch. Enzymatic reaction was carried out for 2 h, followed by cooling in a water bath and a decrease in pH to 4.5 with HCl 1 M. Next, saccharification was performed by adding β-glucoamylase (amyloglucosidase, 300 AGU ml^{-1}, Novozymes Corp., Bagsvaerd, Denmark) at 1.7 ml kg^{-1} starch and mixing in a bath shaker at 60°C and 150 rpm. Time of reaction varied, thus resulting in different treatments: enzymatic treatment 1 (E1), 1 h at 60°C plus 39 h at 40°C; enzymatic treatment 2 (E2), 3 h at 60°C plus 37 h at 40°C; and enzymatic treatment 3 (E3), 6 h at 60°C plus 34 h at 40°C. After saccharification, the enzyme was inactivated by heating for 5 min in a bath at 80°C.

Fermentation studies

Fermentations were conducted in batch mode in 500 ml flasks containing an effective reaction volume of 250 ml and either 60, 80 or 90 g l^{-1} CF solutions. Hydrolyzed CF solutions were autoclaved at 121°C for 20 min; on cooling at room temperature, filter-sterilized P2 medium stock solutions (buffer: KH_2PO_4, 50 g l^{-1}; K_2HPO_4, 50 g l^{-1}; ammonium acetate, 220 g l^{-1}; vitamin: *para*-aminobenzoic acid, 0.1 g l^{-1}; thiamin, 0.1 g l^{-1}; biotin, 0.001 g l^{-1}; mineral: $MgSO_4 \cdot 7H_2O$, 20 g l^{-1}; $MnSO_4H_2O$, 1 g l^{-1}; $FeSO_4 \cdot 7H_2O$, 1 g l^{-1}; NaCl, 1 g l^{-1}, Jesse et al. 2002) were added (10 ml each to 970 ml of hydrolyzed CF solutions), followed by transferring the medium to the flasks. Flasks were sealed with rubber stoppers and air was removed by atmosphere-displacing with a N_2 flux (~69 kPa), applied for 20 min over the medium. To create anaerobic conditions in the media, flasks containing medium were kept in anaerobic jars (HP11, Oxoid, Cambridge, UK) for 48 h prior to inoculation. Anaerobic conditions in

the jar and hence in the medium were created by Anaero-Gen AN35 envelopes (Oxoid). Batch cultures were initiated by adding 5% (v/v) inoculum (Parekh et al. 1998), prepared as previously described. Flasks were incubated under orbital agitation conditions (150 rpm) for 96 h at 35 or 40°C, depending on experiment. pH was not controlled in fermentations, however, it was initially adjusted to 4.5 in all cultures. By the end of the incubation period, samples from the flasks were centrifuged at 3000 rpm for 30 min and the supernatant was filtered (nitrocellulose membranes, 0.22 μm pore diameter) for analytical determinations. Solvent yield was evaluated with a 2^3 factorial design; the study variables were fermentation temperature (x_1, 35 and 40°C), initial substrate concentration (x_2, 60 and 80 g l^{-1}) and hydrolysis strategy (x_3, CT and E1 treatments). Final concentrations of the individual and total solvents in the fermentation broth were considered as response variables. The second experimental design was a two-block design with three treatments (3 × 2) with repetition. The study variables were initial substrate concentration (x_2: 60 and 90 g l^{-1}) and hydrolysis strategy (x_3: E2, E3 and CT); variable responses included the aforementioned, plus the yields of glucose and reductive sugars after hydrolysis. In this second design temperature was fixed at 40°C.

Analytical methods

ABE were measured using a 5890 Hewlett Packard gas chromatograph (Hewlett Packard, Avondale, PA, USA) equipped with a flame ionization detector (FID) and a 30 m × 0.25 mm column (Supelco SPB-5, Bellefonte, PA, USA). Glucose and total reducing sugar concentration were determined according to the glucose oxidase enzymatic Trinder method and the Nelson-Somogyi method, respectively (Sadavisam and Manickam 1996). Productivity was calculated as the ABE concentration achieved (g l^{-1}) divided by the fermentation time. Sugar production yield was defined as the percentage (w/w) of sugar (reducing sugars or glucose) produced after hydrolysis respect to the theoretical initial starch concentration.

Results and discussion

The performance of cassava as a substrate for ABE production was investigated throughout the analyses of the effect of different variables and their interactions. The nutritional composition of the CF produced (total carbohydrates 86.7%; protein 1.3%; fat 0.2%; ashes 2.4%; humidity 9.4%) included a remarkable starch content of 81.2%, higher than that reported for other substrates such as corn flour (Gu et al. 2009). This feature, in addition to its low cost, makes cassava a promising candidate substrate for large-scale fermentation processes aimed at producing ABE (Gu et al. 2009).

Influence of temperature, hydrolysis strategy and initial substrate concentration on solvent yield: factorial design 2^3

The first experimental design was a two-level, three-variable factorial design to determine the best conditions for the production of solvents in ABE fermentation. The yields of acetone, butanol and ethanol for every combination of variables are shown in Table 1.

Figure 1 shows the Pareto charts of the significant effects of the study variables and their interactions on ABE production. Statistical analyses showed that temperature of the process and the initial concentration of CF had significant effects on butanol production, with positive and negative signals, respectively (Fig. 1a), while hydrolysis strategy had no significant effect. Accordingly, an increase in butanol production was observed when rising the temperature from 35 to 40°C. Likewise, initial CF concentration, which exerted the greatest influence on production, showed a negative influence on butanol production at its high level; such increase in solvent production when employing 60 g l^{-1} instead of 80 g l^{-1} indicates that high substrate loads exert an inhibition on *C. beijerinckii*. Substrate inhibition has been demonstrated for this strain in glucose media, being reflected especially as an increase of lag phase when surpassing 100 g l^{-1}, and as lower average rates of cell growth and maximum cell concentration when surpassing 200 g l^{-1} (Qureshi and Blaschek 2001b).

Interactions temperature-CF concentration (x_1x_2, negative effect) and temperature-hydrolysis strategy (x_1x_3, positive effect) had also significant effects. As it is shown in Fig. 1e, the highest change in butanol production occurred when both low CF concentration and high temperature levels were employed, while temperature had little effect when high loads of CF were used. These conditions may promote solvent production since they lead to a reduction in possible substrate inhibition over microbial growth, and approach to the optimal temperature for *C. beijerinckii*. Similarly, as observed in Fig. 1f, butanol production was favored when temperature was fixed at the high level (40°C) and the enzymatic treatment (E1) was employed. This finding could be due to a higher degree of selective substrate hydrolysis by enzymatic means, if compared to the acid treatment. However, practically no difference was obtained when changing the temperature in the case of the chemical hydrolysis. The findings regarding the better performance of fermentation at 40°C are remarkable, since most ABE processes with this strain have been carried out at temperatures ranging from 30 to 35°C (Formanek et al. 1997; Parekh et al. 1998, 1999; Qureshi

Table 1 Experimental plan for factorial design 2^3 performed for CF fermentation by *C. beijerinckii* and results of response variables in terms of ABE production

Run	Study variables			Response variables			
	Temperature (x_1, °C)	CF concentration (x_2, g l^{-1})	Hydrolysis strategy (x_3)	Butanol (g l^{-1})	Ethanol (g l^{-1})	Acetone (g l^{-1})	Total solvents (g l^{-1})
1	35	60	CT	21.87	14.58	0.01	36.46
2	40	60	CT	26.73	1.70	7.19	35.62
3	35	80	CT	27.54	20.25	0.01	47.80
4	40	80	CT	17.82	6.32	0.01	24.15
5	35	60	E1	10.53	2.75	0.01	13.29
6	40	60	E1	31.59	10.21	2.69	44.49
7	35	80	E1	19.44	8.91	0.01	28.36
8	40	80	E1	22.68	2.84	8.14	33.66
9	35	60	CT	19.44	1.54	6.87	27.85
10	40	60	CT	25.92	3.56	11.85	41.33
11	35	80	CT	20.25	9.72	8.69	38.66
12	40	80	CT	18.63	0.01	5.14	23.78
13	35	60	E1	16.20	12.15	0.01	28.36
14	40	60	E1	24.30	7.70	0.01	32.01
15	35	80	E1	11.34	0.01	8.69	20.04
16	40	80	E1	18.63	0.01	7.51	26.15
			Average	20.81	6.39	4.18	31.38

Details regarding fermentation conditions are described in the Materials and methods section

CT chemical treatment; *E1* enzymatic treatment 1

and Blaschek 1999; Qureshi et al. 2001, 2001; Jesse et al. 2002; Ezeji et al. 2004, 2007). It is noteworthy that the same behavior was observed when the total concentration of solvents was considered as the response variable (Fig. 1d), which is in accordance with the fact that butanol represented ~67% of the total solvent production. Meanwhile, when the production of acetone or ethanol was considered as the response variable, none of the design variables or their interactions had a significant effect (Fig. 1b, c).

Considering the average results from all the runs, the process yielded a butanol concentration of 20.81 g l^{-1} in a 96 h period, which corresponded to a productivity of 0.22 g l^{-1} h^{-1}. Likewise, the total solvent production was 31.38 g l^{-1}, with a productivity of 0.33 g l^{-1} h^{-1}. In this work, all the fermentation batches were performed for 96 h, as ABE production has usually stopped by this time (Parekh et al. 1998, 1999; Ezeji et al. 2007). In this respect, research will be conducted to determine the kinetics of solvent production in order to obtain a more suitable productivity value. Results are promising given that a cease in fermentation has been reported at ABE concentrations of 25 g l^{-1} due to toxic effect of the solvents produced (Häggström 1985). Similarly, the yields of acetone and ethanol were 4.18 and 6.39 g l^{-1}, resulting in an ABE ratio of 1.0:5.1:1.6.

Influence of different hydrolysis strategies: randomized block design (3 × 2)

Considering the results obtained in the factorial design regarding the apparent lack of a significant effect of the hydrolysis strategy on solvent production, and based on previous reports (Qureshi et al. 2001), the authors decided to perform further investigation in relation to this variable. Since amylolytic activity of *C. beijerinckii* BA101 is enhanced in the presence of dextrin and maltose (Annous and Blaschek 1991), the substrate was subjected to different pretreatments to obtain a partial hydrolysis of the starch content in the CF. Conditions of enzymatic pretreatments were varied (higher saccharification periods) and determinations of glucose and reducing sugars were added as response variables to estimate the pretreatment efficiency in a randomized two-block design with three treatments (3 × 2) at 40°C.

Results of sugar released by hydrolysis and ABE production are shown in Table 2. Determination of glucose and reducing sugars immediately after hydrolysis, revealed that both enzymatic treatments yielded a similar sugar production, significantly higher than chemical treatment (Fig. 2a). This finding is not strange, considering that acid hydrolysis of starch usually has a low conversion degree, and tends to release non-desirable products, while enzymatic hydrolysis shows a higher conversion rate and its selective character

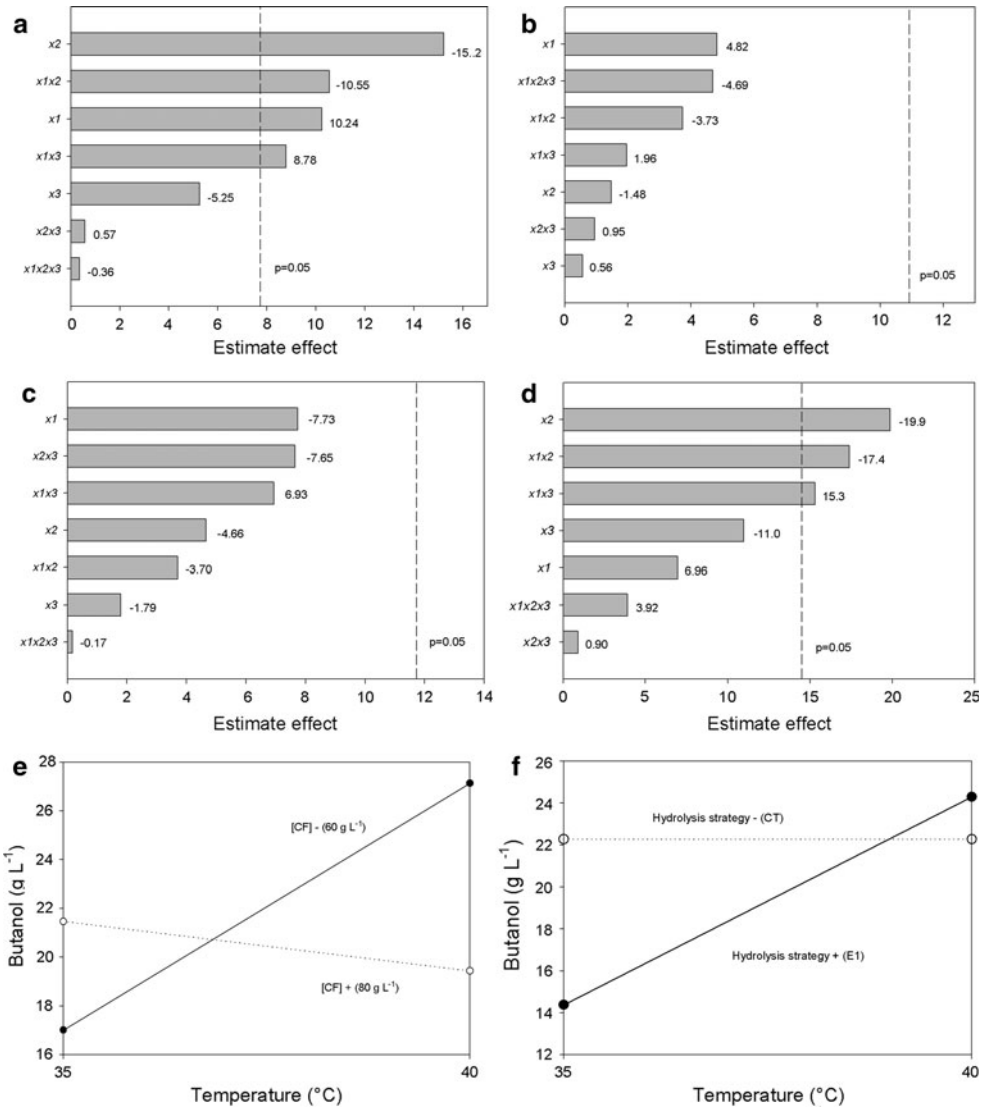

Fig. 1 **a–d** Pareto chart of standardized effects of the response variables and their interactions on the production of butanol (**a**), acetone (**b**), ethanol (**c**) and total solvents (**d**) in cassava flour fermentation by *C. beijerinckii* BA101. The *vertical line* indicates the significance break-down ($P = 0.05$). x_1: temperature; x_2: CF concentration; x_3: hydrolysis strategy; x_1x_2: interaction temperature-CF concentration; x_1x_3: interaction temperature-hydrolysis strategy; x_2x_3: interaction CF concentration-hydrolysis strategy; $x_1x_2x_3$: triple interaction. **e–f** Contrast diagram of significant interactions obtained from the factorial design 2^3 on butanol production. Temperature-initial CF concentration (**e**) and temperature-hydrolysis strategy (**f**). [*CF*] CF concentration, *CT* chemical treatment, *E1* enzymatic treatment 1

reduces by-product formation. Comparing the enzymatic pretreatments, E3 was slightly more efficient to produce sugars than E2, which could be explained by the higher period of saccharification employed in E3.

Regarding ABE production, butanol is the only solvent whose concentration was significantly affected ($P < 0.05$) when fermentation was carried out at different CF concentrations, with the same substrate inhibition effect previously described. However, in this case, important differences were observed depending on the pretreatment strategy (Fig. 2b). Thus, enzymatic treatments E2 and E3 yielded higher butanol (but not acetone or ethanol) concentrations than the acid treatment CT, finding which seems to be related to the more efficient sugar production obtained pre–fermentation by enzymatic means. Moreover, residual enzymatic saccharification simultaneous to fermentation, and/or the enhancing of bacterial amylolytic activity due to higher sugar concentrations might have taken place in the enzymatic treatments. Although CT runs resulted in lower butanol concentrations, it is remarkable that no strong constraint effect was obtained, as acid or alkali pretreatment of some agricultural residues usually results in inhibition of cell growth and butanol production (Qureshi et al. 2007, 2008). Despite the hyperamylase–producing character of *C. beijerinckii* BA101, previous analyses without pretreatment of the substrate resulted in poor solvent production (B: 3.89; E: 0.61; A: 0.013 g l^{-1}).

The average results of the different runs (Table 2) showed a production of 27.28 g l^{-1} and a productivity of 0.284 g l^{-1} h^{-1}, slightly lower than averages in the factorial design. Likewise, the yields of butanol, ethanol and acetone were 17.39, 5.80 and 4.09 g l^{-1} (ABE ratio, 1.0:4.8:1.6). Results are considered as successful, since the only previous report of ABE production by fermentation in a cassava-based medium yielded 13.2 and 19.4 g l^{-1} of butanol and total solvents, respectively (Gu et al. 2009).

Table 2 Experimental plan for design of treatments and blocks performed for CF fermentation by *C. beijerinckii* and results of response variables in terms of glucose, reducing sugar and ABE

Run	Study variables		Response variables					
	CF concentration (x_2, g l^{-1})	Hydrolysis strategy (x_3)	Sugar production yield (%)		Butanol (g l^{-1})	Ethanol (g l^{-1})	Acetone (g l^{-1})	Total solvents (g l^{-1})
			Reducing sugar	Glucose				
1	60	E2	8.99	2.5	20.42	6.89	3.95	31.26
2	60	E2	9.10	2.4	20.92	6.97	3.40	31.29
3	90	E2	9.09	2.6	16.12	6.24	6.16	28.52
4	90	E2	8.92	2.5	16.69	3.24	4.27	24.2
5	60	E3	9.12	2.7	23.98	8.91	0.01	32.9
6	60	E3	9.07	2.6	20.98	10.29	3.79	35.06
7	90	E3	9.05	2.5	20.05	0.44	13.62	34.11
8	90	E3	9.16	2.6	18.74	0.01	3.71	22.46
9	60	CT	6.06	1.0	14.17	4.37	1.98	20.52
10	60	CT	6.03	1.1	13.78	6.92	0.11	20.81
11	90	CT	6.11	1.0	10.98	7.74	3.83	23.96
12	90	CT	6.16	1.0	11.89	7.64	4.27	23.8
		Average	8.072	2.049	17.39	5.80	4.09	27.28

Details regarding fermentation conditions are described in the Materials and methods section

E2 enzymatic treatment 2; *E3* enzymatic treatment 3; *CT* chemical treatment

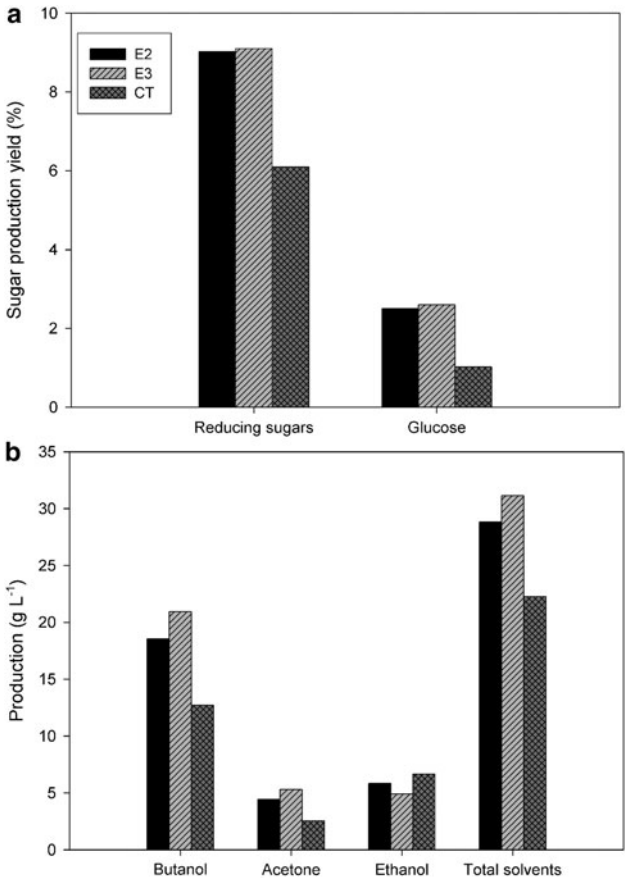

Fig. 2 Effect of hydrolysis strategy on sugar production yield (**a**) and solvent production (**b**) in the randomized block design. *E2* enzymatic treatment 2, *E3* enzymatic treatment 3, *CT* chemical treatment

Although higher productivities have been obtained with other substrates (Qureshi et al. 2007) and taking into account that our values are calculated on the basis of 96 h, the productivity here reported surpasses the expected when working with starch-based substrates (0.1-0.3 g l^{-1} h^{-1}, Häggström 1985). Moreover, the final concentration of ABE achieved in both experimental designs was higher than previously reported for *C. beijerincki* BA101 in batch systems with diverse substrates (Qureshi et al. 2001, 2007, 2008; Jesse et al. 2002). Further research should be carried out to scale up ABE fermentation with CF and to determine the presence of fermentation inhibitors, usually contained in agricultural hydolysates (Ezeji et al. 2007). These promising results, in addition to the ease of cultivation and relatively cheaper price than high value compounds such as glucose or maltodextrins, suggest that cassava could be a suitable substrate for industrial production of ABE.

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