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Optimization of butanol production from tropical maize stalk juice by fermentation with *Clostridium beijerinckii* NCIMB 8052

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ABSTRACT

Mixed sugars from tropical maize stalk juice were used to carry out butanol fermentation with *Clostridium beijerinckii* NCIMB 8052. Batch experiments employing central composite design (CCD) and response surface methodology (RSM) optimization were performed to evaluate effects of three factors, i.e. pH, initial total sugar concentration, and agitation rate on butanol production. Optimum conditions of pH 6.7, sugar concentration 42.2 g/L and agitation rate 48 rpm were predicted, under which a maximum butanol yield of 0.27 g/g-sugar was estimated. Further experiments demonstrated that higher agitation facilitated acetone production, leading to lower butanol selectivity in total acetone–butanol–ethanol (ABE). While glucose and fructose are more preferable by *C. beijerinckii*, sucrose can also be easily degraded by the microorganism. This study indicated that RSM is a useful approach for optimizing operational conditions for butanol production, and demonstrated that tropical maize, with high yield of biomass and stalk sugars, is a promising biofuel crop.

1. Introduction

Bio-butanol produced from renewable resources through microbial fermentation is of great interest, because it not only can be used as an important renewable fuel that has various advantages over ethanol, but also has vast application as a chemical feedstock in many industries (Dürre, 2007). Bio-butanol has been produced through the acetone–butanol–ethanol (ABE) fermentation using the solventogenic clostridia. The cost of substrate is an important part for the overall cost of bio-butanol production (Qureshi and Blaschek, 2001a). Inexpensive and easily-degradable feedstocks are desirable for the ABE fermentation. Tropical maize, a hybrid corn variety made by crossing temperate by tropical adapted parents, is a high energy crop yielding large amounts of biomass and stalk sugar with potentially valuable use as a biofuel crop. The mixed sugars obtained from tropical maize stalk juice are composed of high concentrations of sucrose, glucose and fructose, which are all easily degraded during the microbial fermentation processes.

On the other hand, ABE fermentation is a very complex process that is influenced by many factors. Proper pH control is essential for the fermentation to shift to solventogenesis and produce a high yield of butanol (Jones and Woods, 1986; Maddox et al., 2000); while a low sugar concentration in the feedstock may result in reduced cell growth and unfavorable solvent production, a high sugar concentration can result in substrate inhibition, which may inhibit cell growth and cause failure of fermentation (Ezeji et al., 2003, 2005); suitable agitation rate can facilitate mixing of the substrates and products, enhancing substrate accessibility and products distribution, but high agitation may adversely impact the fermentation, and lead to unnecessary waste of energy and poor industrial economics. A number of studies have been carried out to investigate the effects of different factors on butanol production (Geng and Park, 1993; Nishio et al., 1983; Salleh et al., 2008; Soni et al., 1992; Welsh and Veliky, 1984). However, these studies examined only one or two factors at a time. Since biological butanol production is affected by two or more factors simultaneously, a multi-factorial experimental design approach is required.

Response surface methodology (RSM) is a statistical method useful for evaluating the relative significance of several independent variables, understanding the interactions of the various parameters affecting the process, and hence determining optimal conditions for desirable responses. RSM has the advantage of reducing the number of experiments required and making it easy for overall data analysis (Bezerra et al., 2008). RSM has demonstrated its effectiveness for the optimization of many complex processes in chemical engineering (dos Santos et al., 2005), food

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sciences (Li and Fu, 2005), wastewater treatment (Wang et al., 2007), biological fermentation (Vishwanatha et al., 2010), etc. Therefore, the main objective of this study was to investigate the effects of pH, initial total sugar concentration and agitation rate on ABE fermentation using RSM and thereby try to determine the optimum conditions for maximizing butanol yield. In addition, the potential use of mixed sugars obtained from tropical maize stalk juice as a biofuel feedstock was evaluated.

2. Methods

2.1. Raw materials and reagents

Tropical maize was bred at the Dudley Smith Farm at University of Illinois at Urbana-Champaign (UIUC). The stalks were harvested and squeezed to obtain fresh juice. The juice was autoclaved at 0.15 MPa and 121 °C for 15 min and stored at 4 °C before it was used. The sugar content of the fresh juice was analyzed as (in g/L): sucrose, 95.4; glucose, 23.8; fructose, 7.5. There were also many other mineral chemicals presented in the juice which were supplementary to the bacterial culture growth (in g/L, N: 0.64; P: 0.13; K: 1.01; Na: 0.0013; Ca: 0.16; Mg: 0.27; Fe: 0.042; Mn: 0.0011; S: 0.034).

Other chemicals and reagents were obtained from either Sigma (St. Louis, MO) or Fisher Scientific, Inc. (Hanover Park, IL) and were of analytical grade.

2.2. Bacterial culture and fermentation experiment

Laboratory stocks of *Clostridium beijerinckii* 8052 spores were stored in sterile H_2O at 4 °C. Spores were heat-shocked at 80 °C for 10 min, followed by cooling on ice for 5 min. The heat-shocked spores were inoculated into tryptone–glucose–yeast extract (TGY) medium containing 30 g/L tryptone, 20 g/L glucose, 10 g/L yeast extract and 1 g/L L-cysteine at a 1% inoculum level. The TGY culture was incubated at 35 ± 1 °C for 12–14 h in an anaerobic chamber under N_2:CO_2:H_2 (volume ratio of 85:10:5) atmosphere. Subsequently, actively growing culture was inoculated into the broth containing various concentrations of autoclaved tropical maize stalk juice, 1 g/L yeast extract, and filter-sterilized P2 medium (Qureshi et al., 2001) in a Sixfors bioreactor system (Infors AG, Bottmingen, Switzerland). Oxygen-free nitrogen was flushed through the broth to initiate anaerobiosis until the culture initiated its own gas production (CO_2 and H_2). Initial pH of the fermentation broth was adjusted using 2 M NaOH or HCl. Temperature was controlled at 35 ± 1 °C. Various agitation rates were employed for mixing. During the course of fermentation, 2 ml culture aliquots were collected for product concentration quantification.

2.3. Experimental design

RSM with a full factorial central composite design (CCD) was employed in this study, as shown in Table 1.The variables were coded according to Eq. (1):

$$x_i = \frac{X_i - X_0}{\Delta X_i} (i = 1, 2, 3, \cdots, k) \quad (1)$$

where x_i is the coded value of the ith test variable; X_i is the uncoded value (real value) of the ith test variable; X_0 is the value of X_i at the central point of the investigated range, and ΔX_i is the step size of the ith test variable. pH (X_1), initial total sugar concentration (X_2) and agitation rate (X_3) were chosen as the three independent factors, while final butanol yield (Y) as the response variable. The central values in the experimental design were selected as pH 6.5, sugar concentration 60 g/L and agitation 100 rpm. OriginPro 8.1 (OriginLab Corporation, Northampton, MA) and Matlab 7.10 (The MathWorks, Inc., Natick, MA) were used for the data analysis.

2.4. Analytical procedures

The mineral composition of the fresh juice was analyzed by the Internal Analytical Services Lab at Illinois State Water Survey (Champaign, IL). The sugar concentration was determined using a Shimadzu (Columbia, MD) high-pressure liquid chromatography system. A Bio-Rad HPX-87P column (Bio-Rad Laboratories, Inc., Hercules, CA) equipped with a guard column (30 × 4.6 mm) was used with a mobile phase of ultrapure water at a flow rate of 0.6 ml/min. The column temperature was kept at 85 °C. A refractive index (RI) detector (Waters Corporation, Milford, MA) set at 35 °C was used for signal detection. ABE, acetic acid, and butyric acid concentrations were quantified using a gas chromatography (GC) (Hewlett Packard, Avondale, PA, USA) equipped with a flame ionization detector (FID), a 1829 × 2 mm glass column (10% CW-20M, 0.01% H_3PO_4, support 80/100 Chromosorb WAW) and an Agilent 7683 series automatic liquid sampler (Agilent Technologies, Inc., Palo Alto, CA). The butanol yield was calculated as grams of butanol produced per gram of sugar utilized, while the butanol productivity was calculated as the butanol produced in g/L of broth divided by the fermentation time in h.

3. Results and discussion

3.1. Optimization of butanol production employing response surface methodology (RSM)

CCD was employed to determine the individual and interactive effects of three parameters on butanol yield. The following response equation was used to correlate the dependent and independent variables.

$$Y_i = a_0 + \sum_{i=1}^{k} a_i x_i + \sum_{i=1}^{k} a_{ii} x_i^2 + \sum_{i}^{k} \sum_{j}^{k} a_{ij} x_i x_j \quad (2)$$

where Y_i is the response; x_i, x_j are the input variables, which influence the response variable Y_i; a_0 is the offset term; a_i is the ith linear coefficient; a_{ii} is the quadratic coefficient and a_{ij} is the ijth interaction coefficient.

The RSM experimental design matrix with three factors at five levels and the experimental results are presented in Table 1. The regression coefficient, standard error, student's test t values, and significance level are summarized in Table 2. The t-test value indicates the significance of the regression coefficient. From Table 2, the linear coefficients a_1 and a_2, the quadratic coefficient a_{11}, as well as the interaction coefficient a_{12} are all significant at a 5% significance level. Therefore, the linear effect of pH (a_1) and sugar concentration (a_2), the quadratic effect of pH (a_{11}) and the interaction effect between pH and sugar concentration (a_{12}) are the most influential factors. In addition, a_2 and a_{11} were less than zero, indicating negative effects of these parameters on butanol yield. On the other hand, the linear and quadratic effects of agitation (a_3 and a_{33}), as well as the interactive effects of agitation with pH and sugar concentration (a_{13} and a_{23}) on butanol yield were all slight, as indicated by the large P-values.

Fisher's statistical test for analysis of variance (ANOVA) was used to evaluate the quality of the regression (Table 3). The regression statistics showed that the model represented an accurate representation of the experimental data, as the computed $F_{statistic}$ (10.29) is much larger than $F_{0.05,9,10}$ (3.02). In addition, the small P-value for the regression in Table 3 also indicated the adequacy of the model.

Table 1
Central composite design (CCD) and response results for the butanol production.

Run	Coded values			Real values			Butanol yield (g/g-sugar)
	pH	Sugar concentration	Agitation	pH	Sugar concentration (g/L)	Agitation (rpm)	
1	1	1	1	7.0	80	150	0.26
2	1	1	−1	7.0	80	50	0.26
3	1	−1	1	7.0	40	150	0.24
4	1	−1	−1	7.0	40	50	0.26
5	−1	1	−1	6.0	80	50	0.19
6	−1	1	1	6.0	80	150	0.20
7	−1	−1	1	6.0	40	150	0.25
8	−1	−1	−1	6.0	40	50	0.25
9	2	0	0	7.5	60	100	0.23
10	−2	0	0	5.5	60	100	0.09
11	0	2	0	6.5	100	100	0.20
12	0	−2	0	6.5	20	100	0.25
13	0	0	2	6.5	60	200	0.24
14	0	0	−2	6.5	60	0	0.24
15	0	0	0	6.5	60	100	0.25
16	0	0	0	6.5	60	100	0.26
17	0	0	0	6.5	60	100	0.26
18	0	0	0	6.5	60	100	0.26
19	0	0	0	6.5	60	100	0.25
20	0	0	0	6.5	60	100	0.26

Table 2
Statistics for the regression of the optimization model.

Coefficient	Value	Standard error	Student's *t*	*P*
a_0	0.26	0.0069	37.44	<0.001
a_1	0.026	0.0044	5.89	<0.001
a_2	−0.012	0.0044	−2.73	0.021
a_3	−0.00063	0.0044	−0.14	0.89
a_{11}	−0.023	0.0035	−6.48	<0.001
a_{22}	−0.0063	0.0035	−1.80	0.10
a_{33}	−0.0025	0.0035	−0.72	0.49
a_{12}	0.016	0.0062	2.64	0.025
a_{13}	−0.0038	0.0062	−0.61	0.56
a_{23}	0.0038	0.0062	0.61	0.56

Table 3
ANOVA for the regression.

Source of variation	Degree of freedom	Sum of squares	Mean square	$F_{statistic}$	Prob > *F*
Model	9	0.028	0.0031	10.29	0.00056
Residual	10	0.0030	0.00030		
Total	19	0.031			
R^2 = 0.90					

The optimum conditions for maximum butanol yield, calculated by setting the partial derivatives of Eq. (2) to zero with respect to the corresponding variables, were pH 6.7, a total sugar concentration of 42.2 g/L and an agitation rate of 48 rpm. The maximum response value for butanol yield was estimated as 0.27 g/g-sugar.

With butanol yield as the response, the three-dimensional response surfaces and two-dimensional contour plots are shown in Figs. 1–3. These figures demonstrated the relative effects of two variables on the butanol yield with the third kept constant. In Fig. 1, the butanol yield generally increased to a peak value with the increase in pH and the decrease in the sugar concentration, and then decreased with the further increase in pH or decrease in sugar concentration. The variation of pH is relatively more important than that of sugar concentration on butanol yield. The two-dimensional contour plot (Fig. 1b) shows a symmetrical mound shape with an axis of symmetry parallel to the diagonal, indicating the significant interactive effect between pH and sugar concentration. Fig. 2 indicated that the variation of pH influenced the butanol yield greatly across the test range, while the effect of agitation was trivial at most of the time. Agitation only relatively sensitively interacted with pH around the approximate optimum pH area. As

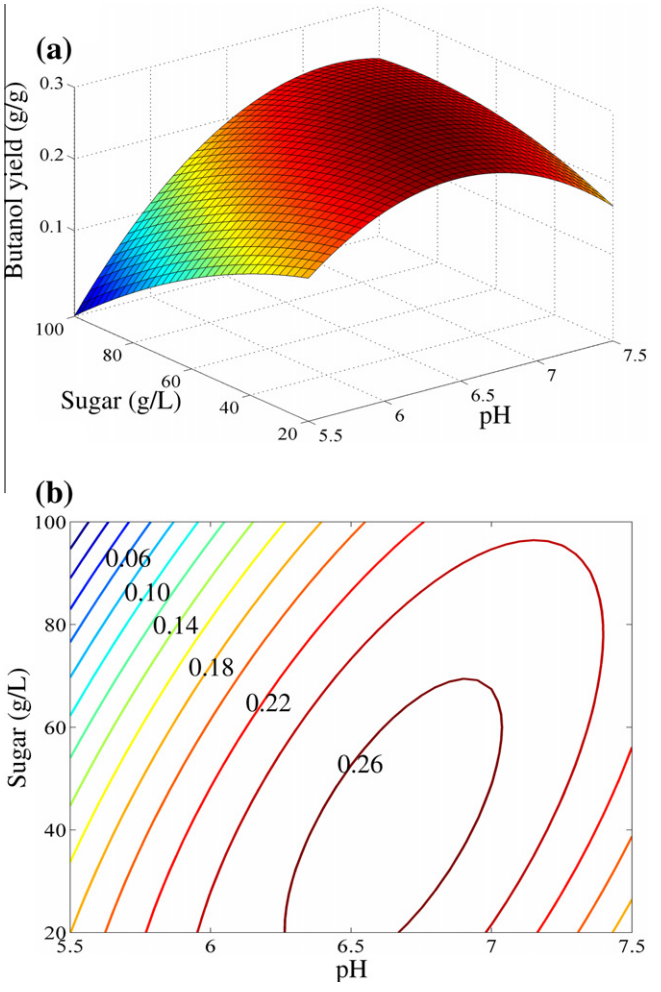

Fig. 1. Response surface and contour plots for butanol yield: effects of pH and sugar concentration (agitation rate was fixed at 48 rpm).

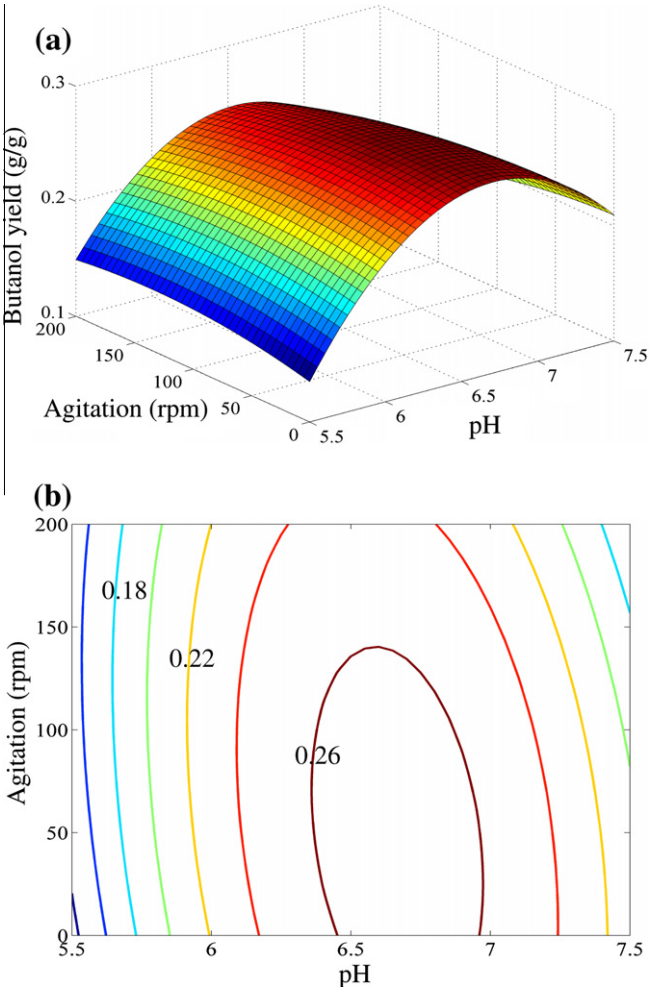

Fig. 2. Response surface and contour plots for butanol yield: effects of pH and agitation rate (sugar concentration was fixed at 42.2 g/L).

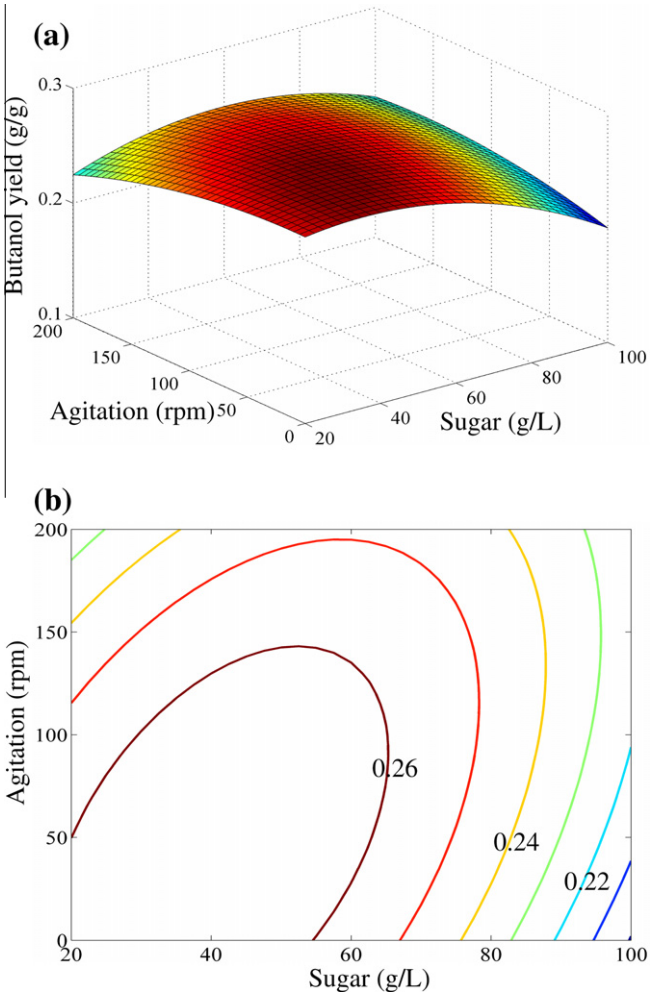

Fig. 3. Response surface and contour plots for butanol yield: effects of sugar concentration and agitation rate (pH was fixed at 6.7).

shown in Fig. 3, the variation of sugar concentration is relatively more important than that of agitation on butanol yield. In addition, it is likely that the interactive effect between sugar concentration and agitation is more important at lower sugar concentration levels than at higher levels. When sugar concentration is low, proper agitation may expedite the substrate distribution and facilitate greater sugar availability. While sugar concentration is high, agitation may not be as important since there is excess substrate available for the cell culture. Generally, the effects of sugar concentration and agitation on butanol yield are not as critical as that of pH, as indicated by a relatively flat response surface shown in Fig. 3a. The order of importance of the three variables on butanol yield is: pH > sugar concentration > agitation.

In the biphasic ABE fermentation, the pH of the medium has long been recognized to be very important for optimum solvent production (Jones and Woods, 1986). During acidogenesis, the rapid formation of acids causes a decrease in pH. Solventogenesis starts when the pH reaches a "break point", after which acids are reassimilated and butanol and acetone are produced. It was assumed that a low pH is a prerequisite for solvent production (Kim et al., 1984). However, in a poorly buffered medium, if the pH decreases below 4.5 before sufficient acids are produced, solventogenesis may not take place or terminate suddenly, which is known as "acid crash" (Maddox et al., 2000). Therefore, in a pH-uncontrolled ABE fermentation, the initial pH is extremely important to assure the switch to solventogenesis and high yield of butanol. During an ABE fermentation with *C. beijerinckii* BA101 in P2 medium, the fermentation was initiated at a pH value near 6.8 and then the pH fell to a lower value (5.0–5.5), which triggered solventogenesis and resulted in a proper butanol production (Qureshi et al., 2008). On the other hand, substrate inhibition is a common problem during solvent fermentation, which may cause long lag phase and make fermentation unsuccessful (Ezeji et al., 2005). A high sugar concentration of 160 g/L was found to be toxic to *C. beijerinckii* BA101 (Ezeji et al., 2003), and cell growth was severely inhibited at above 158 g/L glucose (Qureshi and Blaschek, 2001b). While *C. acetobutylicum* P262 was found to be able to tolerate more than 227 g/L lactose present in whey permeate (Qureshi and Maddox, 2005).

3.2. Confirmation experiments

In order to confirm the validity of the optimization strategy, confirmation experiments were conducted with two replicates under the optimized conditions described above. The butanol yield was obtained as 0.27 g/g-sugar (which is the same as that predicted using RSM), with a high butanol productivity of around 0.30 g/L h. This validated that the RSM approach was effective for optimizing the operational conditions for the butanol fermentation process.

A comparison of the butanol production in this study with those from the literature for butanol fermentation with solventogenic clostridia is summarized in Table 4. Different species of solvento-

Table 4
Comparison of butanol production in this study with results from other studies using solventogenic clostridia.

Carbon sources	Butanol yield (g/g-sugar)	Butanol titer (g/L)	Microorganisms	References
Tropical maize stalk juice	0.27	11.5	*C. beijerinckii* 8052	This work
Glucose	NA[a]	11.2	*C. beijerinckii* 8052	Lee et al. (2008)
Glucose	0.21	12.0	*C. acetobutylicum* ATCC 824	Li et al. (2011)
Cassava starch	0.25	16.4	*C. saccharoperbutylacetonicum* N1-4	Thang et al. (2010)
Cassava starch	NA[a]	13.0	*C. acetobutylicum* EA 2018	Gu et al. (2009)
Liquefied corn starch	0.30	13.4	*C. beijerinckii* BA101	Ezeji et al. (2007b)
Gelatinized sago starch	0.26	8.4	*C. acetobutylicum* P262	Madihah et al. (2001)
Packing peanuts hydrolysates	0.22	13.0	*C. beijerinckii* BA101	Jesse et al. (2002)
DDGS hydrolysates[b]	0.21	10.7	*C. acetobutylicum* P260	Wang et al. (2009)
Wheat bran hydrolysates	0.24	8.8	*C. beijerinckii* ATCC 55025	Liu et al. (2010)
Corn stover hydrolysates	0.20	8.3	*C. acetobutylicum* ATCC 824	Wang and Chen (2011)

[a] Not calculated based on per unit of sugar utilized since butyrate or acetate has been added to supplement the medium.
[b] DDGS: distiller's dried grains with solubles.

genic clostridia have been examined with respect to the butanol yield and final titer. Results from this study were comparable to those from studies using easily fermentable carbon sources (such as glucose, cassava starch and liquefied corn starch, etc.). Fermentation with biomass hydrolysates had lower final butanol concentration and yield possibly due to the inhibitors presented in the hydrolysates (Ezeji et al., 2007a). It is worthwhile noting that *Clostridium saccharoperbutylacetonicum* N1-4, which has a hyperamylolytic activity to hydrolyze starch, can produce high level of butanol from the direct fermentation of cassava starch (Thang et al., 2010).

3.3. Effects of agitation on ABE productivity and butanol/acetone ratio

To further investigate the effects of agitation on ABE production, experiments were carried out under the estimated optimum pH (6.7) and sugar concentration (42.2 g/L) but various agitation levels. As shown in Fig. 4, with the increase of agitation from 0 to 150 rpm, the ABE yield increased gradually from 0.35 to 0.39 g/g-sugar, and then increased slightly more with a further increase of agitation rate. While the butanol/acetone ratio decreased dramatically from 2.8 to around 1.5 with the increase of agitation. The decreased fraction of butanol in the total ABE mix led to a decline in butanol yield (from a peak at 48 rpm) with the increase of agitation rate, except for a slight increase at 400 rpm (Fig. 4). This is consistent with the RSM optimization results that the agitation terms had negative effects on the response butanol yield (both a_3 and a_{33} in Table 2 are <0), although these effects were not significant.

During the production of acetone, CO_2 is produced at the same time (Jones and Woods, 1986). In this study, it is speculated that the high agitation rate facilitated the emission of CO_2 out of the fermentation broth and thereby resulted in a favorable carbon flow towards acetone production instead of butanol production. Although the overall ABE yield was increased with the increased levels of agitation, the net butanol yield actually decreased.

3.4. Sugar consumption

Fig. 5 illustrates the mixed sugar (sucrose, glucose and fructose) degradation profiles during the ABE fermentation at a central point condition of RSM experiment. Glucose and fructose started to be degraded right after inoculation of the culture, while sucrose utilization began 14 h after the start of fermentation. During the fermentation process, approximately 70% of the glucose and fructose were utilized, while only about 50% of the sucrose was consumed. The results suggested that the monosaccharides glucose and fructose are more preferable for ABE production by *C. beijerinckii*, but the microorganism also has a good capability to degrade sucrose. The phosphoenolpyruvate-dependent phosphotransferase system (PTS) mediated sucrose transport mechanism has been proposed for *C. beijerinckii* and it has been confirmed that all three components of the sucrose-specific PTS are presented in *C. beijerinckii* 8052 (Tangney et al., 1998). The sucrose uptake by PTS results in an accumulation of intracellular sucrose 6-phosphate. The sucrose 6-phosphate is further metabolized by sucrose-6-phosphate hydrolases, producing glucose 6-phosphate and fructose. Fructokinases then phosphorylate fructose,

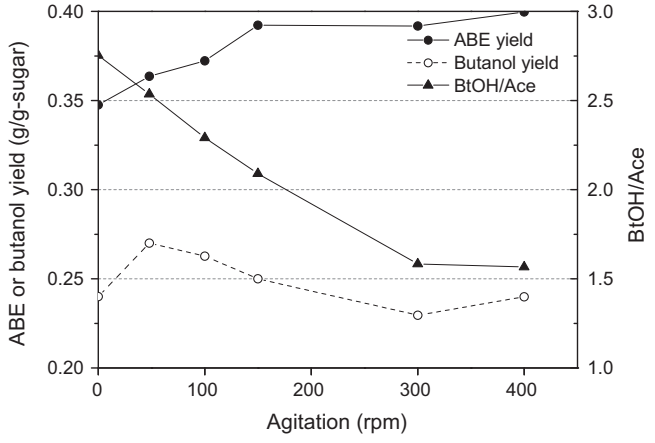

Fig. 4. The effect of agitation on final ABE yield, butanol yield and butanol/acetone (BtOH/Ace in the figure) ratio.

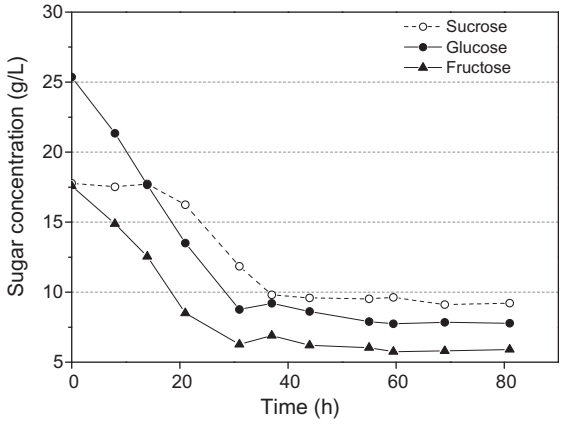

Fig. 5. The degradation profiles of the mixed sugars (sucrose, glucose and fructose) in tropical maize stalk juice during ABE fermentation.

and thus both products from sucrose hydrolysis can be incorporated into the glycolytic pathway (Reid et al., 1999).

Previous studies demonstrated that *C. beijerinckii* had a broad substrate range for ABE fermentation and can degrade many kinds of sugars generated from agricultural residuals and biomass (Ezeji and Blaschek, 2008; Ezeji et al., 2007a). Tropical maize, as a high energy crop yielding large amounts of biomass and stalk sugars, has great potential value used as a biofuel crop.

4. Conclusions

RSM was successfully applied to optimize butanol production from tropical maize stalk juice. A maximum butanol yield of 0.27 g/g-sugar was estimated at the optimum condition of pH 6.7, a sugar concentration 42.2 g/L and an agitation rate 48 rpm. The linear and quadratic effects of pH, linear effect of sugar concentration, and interactive effect between pH and sugar concentration are significant. Higher agitation rates facilitated acetone production, leading to lower butanol fraction in total ABE. While glucose and fructose are more preferable by *C. beijerinckii* for ABE production, sucrose can also be easily degraded by the microorganism.

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