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Full Paper

Response surface optimisation for acetone-butanol-ethanol production from cassava starch by co-culture of *Clostridium butylicum* and *Bacillus subtilis*

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Abstract: Acetone-butanol-ethanol (ABE) production from cassava starch was enhanced by a syntrophic co-culture of *Clostridium butylicum* TISTR 1032 and high amylase producing *Bacillus subtilis* WD 161 without anaerobic pretreatment. The production of amylase and ABE using this co-culture were respectively 16 and 6 times higher than those using the pure culture of *C. butylicum* TISTR 1032. The effect of the medium components on the performance of the co-culture was investigated using response surface methodology (RSM). Among the investigated components, cassava starch and ammonium nitrate contributed a significant effect on the production of amylase and ABE, while yeast extract had less effect. Based on the optimum strategy using RSM, the ABE production by the co-culture was improved 2.2-fold compared with that obtained from the initial condition and with a minimum requirement of nitrogen source.

Keywords: acetone–butanol–ethanol fermentation, *Bacillus subtilis*, cassava starch, *Clostridium butylicum*, co-culture

INTRODUCTION

Energy derived from renewable substrates possesses a number of advantages over fossil-derived energy. These include being renewable, more environmentally friendly and more profitable.

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In addition, the production of bioenergy provides new markets for the agricultural sector and turns agricultural wastes into more valuable products. Thus, it is worthwhile replacing fossil fuels by bioenergy carriers [1]. In addition to ethanol and biodiesel which are currently commonly used, butanol is one of a number of promising energy substances for future use. Compared to ethanol, butanol is more advantageous as it has a higher energy content, is less sensitive to temperature, and requires no modification for use in combustion engines [2]. Butanol is biologically produced along with small amounts of acetone and ethanol by *Clostridium* spp. from renewable materials under strictly anaerobic conditions. This process is called the acetone-butanol-ethanol (ABE) fermentation process. However, the bio-butanol has not been marketed due to its high production cost. The raw material accounts for about 63% of the cost of the fermentation product [3].

The use of starch as the substrate is probably one of the most economically feasible choices due to the low cost and the availability of starch. The ABE production from starch by *Clostridium* actually includes three processes. These are starch hydrolysis by amylolytic enzymes to produce glucose for cell growth, acid (acetic and butyric) production during the acidogenesis phase, and the conversion of these acids into ABE products during the solventogenesis phase. However, starch hydrolysis by *Clostridium* is often less effective due to its low amylase activity. The pre-hydrolysis of starch by commercial enzymes or by acids at high temperature each has its own drawback [1]. Since amylolytic enzymes were determined as a key factor in ABE production from starch, a co-culture of *Clostridium* and another organism in a way that naturally enhances amylolytic activity suggests a possibility of making starch hydrolysis more complete, providing sugar for clostridial growth, and consequently enhancing ABE production. In accord with this concept, a co-culture of *Clostridium* and an amylase producing aerobic *Bacillus* would be more profitable. This is because the *Bacillus* will not only assist the *Clostridium* in substrate hydrolysis, but will also maintain an anaerobic condition by consuming any available oxygen in the culture [4-5]. Thus, there will be less need of pre-hydrolysis of starch and anaerobic pretreatment by addition of a reducing agent and N_2 flushing of the fermentation medium. Thus, this syntrophic co-culture of anaerobic *Clostridium* and aerobic *Bacillus* might also reduce the costs of the biofuel production from starch.

The medium components such as starch concentration, nitrogen source and its content have been reported to have a great influence on ABE production from starch [6]. Although the effects of starch concentration, C/N ratio and ratio of organic/inorganic nitrogen sources on ABE production by a co-culture of *Clostridium* and *Bacillus* have been reported [7], the interactions between the variables were not considered. Response surface methodology (RSM), where the combined effects of all variables are determined through mathematical and statistical inference from experimental design to result analysis, has been applied in systems employing other cultures [8-10]. Only one work [11] employed RSM for the optimisation of ABE production, in which a pure culture of *C. acetobutylicum* P262 was used on sweet potato.

The aim of this study is to use RSM to determine the effect of some of the medium components as well as their interactions on amylase activity and ABE production using a syntrophic co-culture of *Clostridium butylicum* TISTR 1032 and amylase producing *Bacillus subtilis* WD 161 on cassava starch. It is known that starch concentration and a combination of organic-inorganic nitrogen sources are important for the enhancement of amylase activity and ABE production. In this study, yeast extract and ammonium nitrate were used as organic and inorganic nitrogen sources respectively. Yeast extract is well known for providing various amino acids, vitamins, minerals and

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growth factors that promote growth of microorganisms. Ammonium nitrate supports growth and amylase production of *Bacillus* under an anaerobic condition [12]. Thus, the effects of these three factors, namely cassava starch, yeast extract and ammonium nitrate, as well as their optimum levels, were determined using RSM.

MATERIALS AND METHODS

Chemicals and Microorganisms

All chemicals used were of analytical grade and purchased from Fluka Chemical Corporation. *Clostridium butylicum* TISTR 1032 was purchased from Thailand Institute of Scientific and Technological Research (TISTR). The stock culture was maintained in the form of a spore suspension in 25% glycerol and frozen at –20°C. *Bacillus subtilis* WD 161 was a generous gift from Assoc. Prof. Dr. Poonsuk Prasertsan of the Environmental Technology Laboratory, Department of Industrial Biotechnology, Faculty of Agro–Industry, Prince of Songkla University). The stock culture was maintained at 4°C on a nutrient agar slant and subcultured monthly

Inoculum Preparation

C. butylicum TISTR 1032 was anaerobically pre-cultured in a reinforced Clostridia medium (RCM, Oxoid) (1 L RCM contains 10 g meat extract, 5 g peptone, 3 g yeast extract, 5 g glucose, 1 g soluble starch, 5 g sodium chloride, 3 g sodium acetate, and 0.5 g L-cysteine). It was then incubated under static condition at 37°C for 18–24 h. *B. subtilis* WD 161 was aerobically pre-cultured in a nutrient broth (NB) (HiMedia) under shaking condition at 200 rpm and 37°C for 12–18 h.

Fermentation Conditions

For ABE production, B medium was used (1L B medium, pH 6.5, contains 20 g cassava starch, 5 g yeast extract, 2 g NH_4NO_3, 0.5 g KH_2PO_4, 0.3 g $MgSO_4 \cdot 7H_2O$, 0.02 g $MnSO_4 \cdot 7H_2O$, 0.02 g $FeSO_4 \cdot 7H_2O$, and 0.02 g NaCl) [13]. Where noted, the concentrations of cassava starch, yeast extract and ammonium nitrate in B medium were varied to investigate their effects on ABE production. The cultures were established in 120-mL butyl-rubber-sealed serum bottles without anaerobic pretreatment which is normally done by addition of a reducing agent and flushing with N_2 gas over the medium. The working volume of all cultures was 100 mL and the fermentation process was carried out at 37°C. The co-culture was prepared by dispersing 5 mL of inoculum of each organism (6.1×10^4 CFU/mL for *C. butylicum* TISTR 1032 and 3.8×10^7 CFU/mL for *B. subtilis* WD 161) grown as previously described. For comparison purpose, the pure culture was prepared by inoculating 5 mL of inoculum of *C. butylicum* TISTR 1032. All the experiments were carried out at least in duplicate.

Optimisation of Medium Components using RSM

The effect of three variables, viz. cassava starch concentration (x_1), yeast extract concentration (x_2) and ammonium nitrate concentration (x_3), on acetone–butanol–ethanol concentration (ABE) (Y_1), butanol concentration (butanol) (Y_2) and amylase activity (amylase) (Y_3)

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were investigated at three levels (low: –1; medium: 0; and high: +1). Box–Behnken design was employed for the study of interactions between the three variables [14]. Response surface plots for the models were done using the Statistica for Windows version 5.0 to plot the functions of two variables while keeping the other variable at a constant value.

Analytical Methods

Cell growth was determined by measurement of optical density at 660 nm (OD_{660}) using a spectrophotometer (Libra S22, England). During the fermentation period (72 h), a 3.0- ml sample was taken every 12 h and centrifuged at 8000 rpm and 4°C for 25 min. The supernatant was used for determination of ABE, organic acid and residual reducing sugar concentrations and amylase activity. ABE and organic acids were determined using a gas chromatograph (Hewlett Packard) equipped with a glass column (HP–INNOWax polyethylene glycol) and a flame ionisation detector with helium as the carrier gas. The operating conditions were as follows–inlet temperature: 220°C; oven temperature: initial 50°C, ramped up to 115°C at 5°C/min; detector temperature: 270°C [15]. The reducing sugars were estimated by the dinitrosalicylic acid (DNS) method [16]. Briefly, the sample solution (1 mL) was added to DNS solution (3 mL). The absorbance of the solution was measured with a spectrophotometer at 550 nm. The reducing sugars concentration was calculated using a glucose standard calibration curve. Amylase activity was determined by the starch hydrolysis method [17]. The reaction mixture consisted of 1% soluble starch (1.25 mL), 0.2M acetate buffer (pH 5.0) (0.5 mL), and tested sample (0.25 mL). After 10 min of incubation at 50°C, the reaction was stopped by boiling at 100°C for 10 min. The control was carried out in the same manner using a sample pre-inactivated by boiling for 15 min. The liberated reducing sugars were estimated by the DNS method as mentioned above. One unit (U) of amylase is defined as the amount of enzyme that releases one μmole of glucose equivalent per min under the assay condition.

RESULTS AND DISCUSSION

Syntrophic Co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 for ABE Production

The cultivation of the co-culture in this study was performed without anaerobic pretreatment as previously reported [7]. The co-culture of *C. butylicum* TISTR 1032 and high amylase producing *B. subtilis* WD 161 was established for ABE production from cassava starch compared to the pure culture of *Clostridium* itself under condition without anaerobic pretreatment (Figure 1). The medium was composed of 20 g/L cassava starch as carbon source, and 5 g/L yeast extract and 2 g/L ammonium nitrate as organic and inorganic nitrogen sources respectively. As illustrated in Figure 1A, the pure culture of *C. butylicum* TISTR 1032 produced low amounts of acids and ABE from cassava starch since it showed very low amylase activity (1.85 U/mL) and could not utilise cassava starch effectively. On the other hand, the amylase activity produced by the co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 increased 16-fold or up to 30.5 U/mL (Figure 1B). Consequently, the ABE production by the co-culture was enhanced 6-fold or up to 4.01 g/L compared to that of the pure culture of *C. butylicum* TISTR 1032. The high amylase activity in

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Bacillus apparently converts starch to available sugar rapidly, which stimulates the metabolism of *Clostridium* to grow and thus enhances both the ABE production and its rate.

As it has been proved in the previous study [7] that the products from the pure culture of *B. subtilis* WD 161 without anaerobic pretreatment were only ethanol and acetic acid at very low concentrations (<0.2 g/L), it is assumed that the total acids and solvents detected in the product obtained with the co-culture result mostly from the activity of *C. butylicum* TISTR 1032.

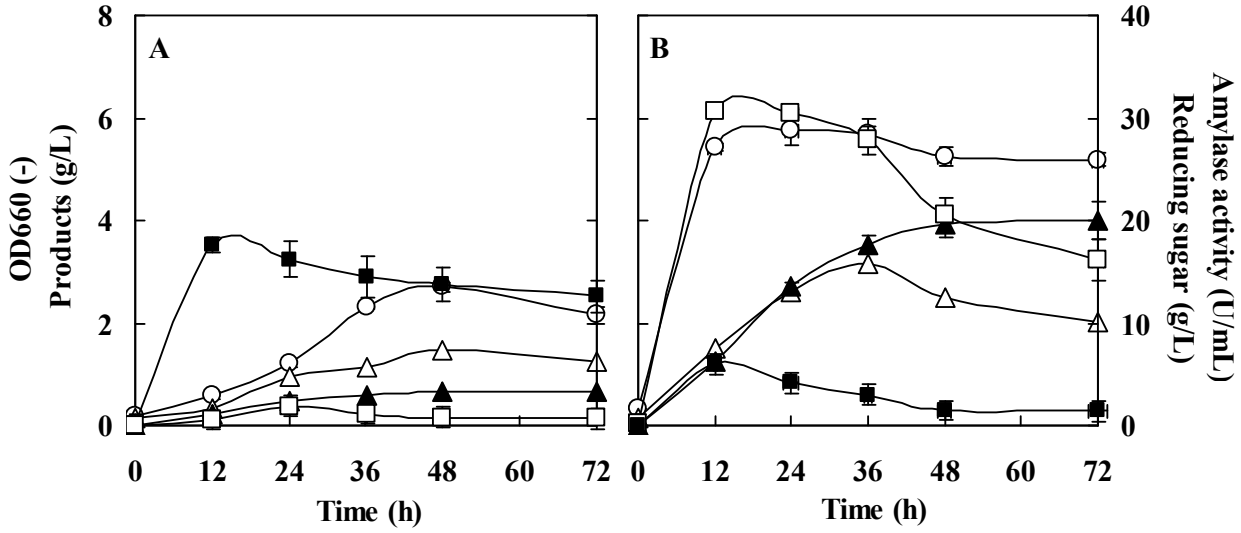

Figure 1. Growth and metabolic activity of pure culture of *C. butylicum* TISTR 1032 (A) and co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 (B) under initial condition (20 g/L cassava starch, 5 g/L yeast extract and 2 g/L ammonium nitrate). Legend: OD_{660} - open circle; amylase activity - open square; reducing sugars - filled square; acids (sum of acetic and butyric acids) - open triangle; ABE - filled triangle.

Response Surface Methodology for Optimising Co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161

The syntrophic co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161 was optimised using RSM. The effects of three variables, i.e. cassava starch concentration (x_1), yeast extract concentration (x_2) and ammonium nitrate concentration (x_3), were investigated. The complete design consisted of a total of 15 trials which contained three replications at the central point for estimating the purely experimental uncertainty variance. The responses observed were ABE concentration (ABE) (Y_1), butanol concentration (butanol) (Y_2) and amylase activity (amylase) (Y_3). The experimental design and respective experimental results are given in Table 1. The regression coefficients (β) and analysis of variances are shown in Table 2.

The response surface analysis was based on multiple linear regressions taking into account the main, quadratic and interaction effects in accordance with the following equation**:**

$$Y = \beta_o + \sum \beta_i x_i + \sum \beta_{ii} x^2_i + \sum \beta_{ij} x_i x_j \quad (1)$$

where Y is the predicted response, x_i and x_j are input variables which influence the response variable Y, β_o is the offset term, β_i is the ith linear coefficient, β_{ii} is the ith quadratic coefficient, and β_{ij} is the ijth interaction coefficient.

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The polynomial equations for ABE (Y_1), butanol (Y_2) and amylase (Y_3) are listed as follows:

$Y_1 = -6.6998 + 0.6820x_1 - 0.0404x_2 + 0.2652x_3 - 0.0078x_1^2 + 0.0011x_2^2 - 0.0086x_3^2 + 0.0018x_1x_2 - 0.0011x_1x_3 - 0.0020x_2x_3$

$Y_2 = -4.2271 + 0.4091x_1 + 0.0614x_2 + 0.2360x_3 - 0.0049x_1^2 - 0.0027\,x_2^2 - 0.0137x_3^2 + 0.0006x_1x_2 + 0.0003x_1x_3 + 0.0008x_2x_3$

$Y_3 = -89.0804 + 6.0833x_1 - 0.7859x_2 + 5.3586x_3 - 0.0784x_1^2 + 0.0247x_2^2 - 0.1536x_3^2 + 0.0541x_1x_2 - 0.0254x_1x_3 - 0.1524x_2x_3$

Table 1. Experimental data for the effects of three variables (cassava starch concentration, yeast extract concentration and ammonium nitrate concentration) with three-level response surface analysis

Trial	Independent variable			Dependent variable		
	Cassava starch (g/L)	Yeast extract (g/L)	Ammonium nitrate (g/L)	ABE (g/L)	Butanol (g/L)	Amylase (U/mL)
	x_1	x_2	x_3	Y_1	Y_2	Y_3
1	1(60.0)	1(20.0)	0(7.0)	4.37	2.50	46.0
2	1(60.0)	-1(5.0)	0(7.0)	5.49	2.17	39.0
3	-1(20.0)	1(20.0)	0(7.0)	3.15	2.89	20.1
4	-1(20.0)	-1(5.0)	0(7.0)	3.20	2.50	25.6
5	1(60.0)	0(12.5)	1(12.0)	3.50	2.00	33.4
6	1(60.0)	0(12.5)	-1(2.0)	2.80	1.80	27.0
7	-1(20.0)	0(12.5)	1(12.0)	2.70	1.75	26.4
8	-1(20.0)	0(12.5)	-1(2.0)	1.92	1.81	15.0
9	0(40.0)	1(20.0)	1(12.0)	9.37	6.12	75.0
10	0(40.0)	1(20.0)	-1(2.0)	8.10	5.60	64.6
11	0(40.0)	-1(5.0)	1(12.0)	8.90	5.80	67.2
12	0(40.0)	-1(5.0)	-1(2.0)	6.70	5.00	36.0
13	0(40.0)	0(12.5)	0(7.0)	9.00	6.00	69.0
14	0(40.0)	0(12.5)	0(7.0)	9.05	5.90	67.3
15	0(40.0)	0(12.5)	0(7.0)	9.13	6.00	66.7

Note: Values in parentheses are uncoded independent variables.
x_1 = cassava starch concentration, x_2 = yeast extract concentration, x_3 = ammonium nitrate concentration, Y_1 = ABE concentration, Y_2 = butanol concentration, Y_3 = amylase activity

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Table 2. Regression of coefficients and analysis of variance of the second-order polynomial for response variables

Coefficient	ABE (g/L) Y_1	Butanol (g/L) Y_2	Amylase (U/mL) Y_3
β_o	- 6.6998*	- 4.2271*	- 89.0804*
Linear			
x_1	0.6820*	0.4091*	6.0833*
x_2	- 0.0404	0.0614	- 0.7859
x_3	0.2652	0.2360	5.3586*
Interaction			
x_1x_2	0.0018	0.0006	0.0541*
x_1x_3	- 0.0011	0.0003	- 0.0254*
x_2x_3	- 0.0020	0.0008	- 0.1524
Quadratic			
x_1^2	- 0.0078*	- 0.0049*	- 0.0784*
x_2^2	0.0011	- 0.0027	0.0247
x_3^2	- 0.0086	- 0.0137	- 0.1536
Variability			
R^2 of model	0.99	0. 97	0.98
F value of model	63.17	4.19	29.95
P >*F*	0.016	0.002	0.032
CV of model	6.7	10.1	10.3

Note: x_1, x_2 and x_3 are cassava starch, yeast extract and ammonium nitrate concentrations respectively.

* Means significant at 5% level

Generally, the adequacy of a model is determined through R^2 (multiple correlation coefficient), CV (coefficient of variation) and *P* values. R^2 value closer to 1 denotes better correlation between the experimental and predicted values. As shown in Table 2, the models for ABE, butanol and amylase are adequate since their R^2 values were found close to 1: 0.99, 0.97 and 0.98 respectively. These indicated that 99%, 97% and 98% of the variability in the response could be explained by the models used for ABE, butanol and amylase respectively. The CV value as the ratio of the standard error of the estimate to the mean value of the observed response indicates the degree of precision with which the experiments are compared. A low reliability of an experiment is usually indicated by a high value of CV (> 20). In the present case, acceptable CV values (6.7, 10.1 and 10.3) are observed for the models of ABE, butanol and amylase respectively, denoting that the experiments performed were reliable. The *P* values (< 0.05) of these three models indicate the significance of the coefficients.

In term of the determination of interactions between the variables, the *P* values can provide an understanding of the pattern of the interactions as well as the effect of each variable on the investigated responses. Further statistical analysis of the effect of each variable in Table 2 shows that only cassava starch concentration (x_1) and its quadratic effect (x_1^2) have a significant effect on all the

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responses (P<0.05). In the case of amylase activity, besides cassava starch concentration, ammonium nitrate (x_3) also has a significant effect (P<0.05). In addition, the interaction terms of x_1x_2 and x_1x_3 are found to be significant for amylase activity (P<0.05). In the work of Bard and Hamdy [11], RSM was employed to investigate the interactive effect of a number of medium components on ABE production by *C. acetobutylicum* P262. The obtained statistical analyses also indicated that the concentration of starch significantly affects the yield and productivity of ABE.

Optimal Conditions for ABE Production

The interaction effects and optimal levels of cassava starch, yeast extract and ammonium nitrate concentrations were determined by plotting the response surface curves. Based on the analysis of variance of the second-order polynomial model of the three investigated variables, yeast extract has the least effect on all responses (Table 2). Thus, the yeast extract concentration was fixed at selected levels (5.0, 12.5 and 20 g/L) and the response surface curves representing the interaction effects of two variables, i.e. cassava starch and ammonium nitrate concentrations, on the production of ABE, butanol and amylase were plotted. (Figures 2–4). The shapes of the response surface curves show a moderately positive interaction between these two variables on the production of ABE, butanol and amylase. Cassava starch obviously affects all the responses more than does ammonium nitrate. The increase in cassava starch from 20 g/L to approximately 40 g/L increases ABE, butanol and amylase production at all selected yeast extract concentrations.

Figure 2 shows that a maximum ABE production is obtainable at a medium concentration of cassava starch (40 g/L) and a considerably high concentration of ammonium nitrate (14 g/L). However, Figure 3 shows that a maximum butanol production is obtainable using a medium concentration of both cassava starch (40 g/L) and ammonium nitrate (8 g/L). When cassava starch concentration is increased more than the optimal level of 40 g/L, reduction in ABE, butanol and amylase is observed (Figures 2–4). This is likely to be due to the high viscosity of the culture medium, which may hinder the mass transfer of enzyme hydrolysis and microbial reactions [6, 20]. It was also reported that a high starch concentration causes a high accumulation of organic acids that would cause toxicity to cells [6]. In addition, increased starch concentration also produces a high amount of glucose or available sugars, which would possibly repress the production of amylase [21].

Since starch hydrolysis is the first step in the production of ABE from starch, the amylolytic enzymes are a key factor in ABE production [6]. As amylase activity in the culture becomes high, starch hydrolysis should be more complete and the sugars for cell growth and ABE production should be more available. Figure 4 depicts the effects of the interaction of cassava starch and ammonium nitrate on amylase activity. The shape of the response surface indicates a a large effect of both variables at low concentration of yeast extract (Figure 4A) while at high concentration of yeast extract (Figure 4C), the amylase activity mostly depends on the concentration of cassava starch. Although highest amylase activity (66 U/mL) is achieved at high concentration of yeast extract (Figure 4C), a considerably high level of amylase activity (61 U/mL) can also be obtained at low concentration of yeast extract by increasing the amount of ammonium nitrate up to 14 g/L (Figure 4A).

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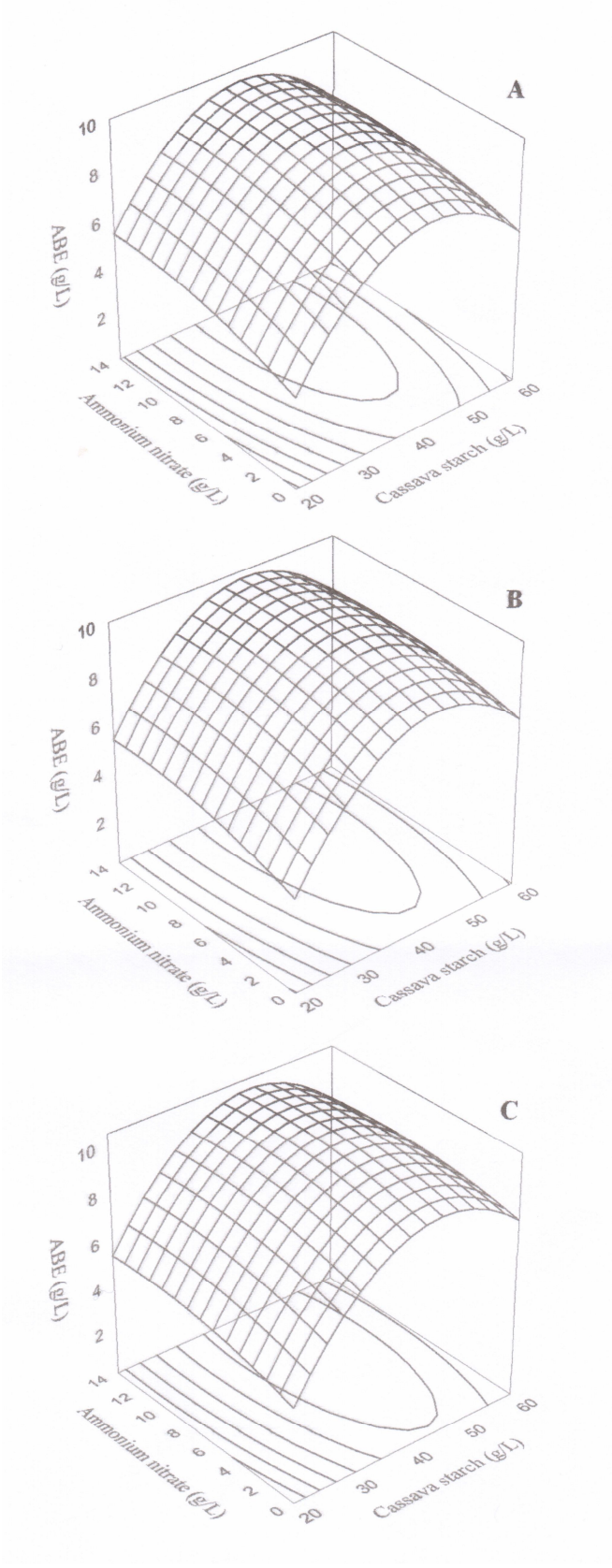

Figure 2. Response surface plots representing the interaction between cassava starch and ammonium nitrate concentrations and their effects on ABE production at given yeast extract concentrations: 5 g/L (A), 12.5 g/L (B) and 20 g/L (C)

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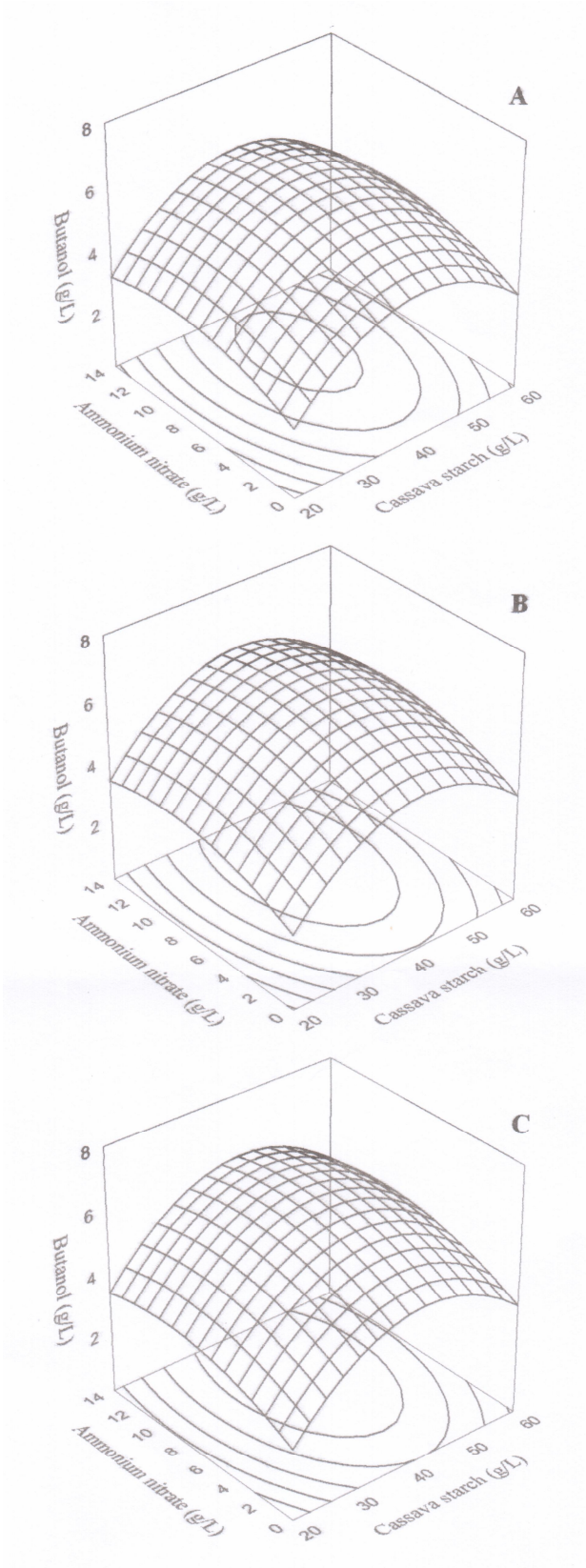

Figure 3. Response surface plots representing the interaction between cassava starch and ammonium nitrate concentrations and their effects on butanol production at given yeast extract concentrations: 5 g/L (A), 12.5 g/L (B) and 20 g/L (C)

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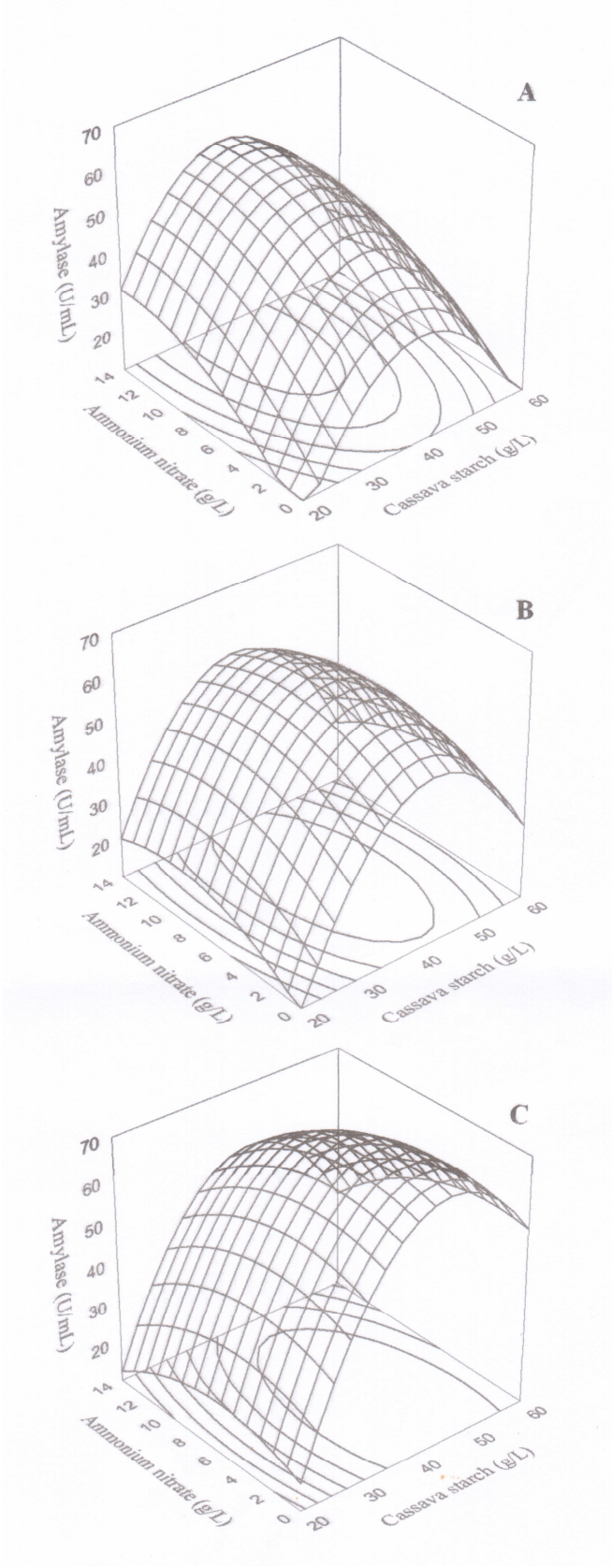

Figure 4. Response surface plots representing the interaction between cassava starch and ammonium nitrate concentrations and their effects on amylase activity at given yeast extract concentrations: 5 g/L (A), 12.5 g/L (B) and 20 g/L (C)

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The increased ABE and butanol production in the co-culture with increasing ammonium nitrate concentration is probably due to the effect of ammonium nitrate on the growth and amylase production of *B. subtilis* WD 161. It was reported that *Bacillus* can grow under an anaerobic condition in the presence of ammonium nitrate since the nitrate ions from ammonium nitrate can replace oxygen as an electron acceptor in the absence of oxygen [12, 22]. Although amylase production increases with increasing concentration of ammonium nitrate, the nitrate concentration higher than 8 g/L does not show a significantly enhanced effect on amylase activity, and consequently on either ABE or butanol concentration.

From RSM, establishing that the ABE and butanol production is mainly influenced by the concentrations of cassava starch and ammonium nitrate rather than yeast extract is important information. This makes it possible to develop a strategy to maximise ABE and butanol production with a minimum requirement of costly organic nitrogen source. From Figures 2 and 3, it can be seen that the maximum ABE and butanol concentrations obtained at all levels of yeast extract are not significantly different. Thus, an optimum condition for both ABE and butanol can be determined by varying only two variables, i.e. cassava starch and ammonium nitrate, and fixing yeast extract concentration at a minimum level (5 g/L). When the concentration of starch is fixed at the optimal level (40 g/L), the concentration of ammonium nitrate can be determined at a range so as to achieve an adequate amylase activity for enhancing ABE and butanol production.

In ABE fermentation, acetone, butanol and ethanol are normally produced in the ratio of 3 : 6 : 1. Increasing ABE concentration without any reduction in the proportion of butanol is the target of most of the optimising work on ABE fermentation process. When butanol is present as a major product in the culture, its recovery process is much easier [3]. To optimise both ABE and butanol production using RSM, a superimposing of the optimal area for ABE and butanol production using Lotus Freelance Graphics at 5 g/L yeast extract concentration was carried out. The optimal points for both ABE and butanol production were in the centroid of the overlapping area as shown in Figure 5. The superimposed contour plots reveal that the optimum conditions for the production of ABE and butanol cover a large range. The central point of this area was selected for maximising both ABE and butanol production. Thus, the optimum condition for effective ABE production was: 40 g/L cassava starch, 5 g/L yeast extract and 8 g/L ammonium nitrate, at which an output of 9.43 g/L ABE, 5.80 g/L butanol and 55 U/mL amylase was predicted. The optimum condition was then experimentally tested and the results obtained are shown in Table 3.

Table 3. Predicted and observed values for optimal production of ABE, butanol and amylase

	Predicted value	Observed value ± SD	CV
ABE (g/L)	9.43	9.02 ± 0.17	1.92
Butanol (g/L)	5.80	5.60 ± 0.13	2.37
Amylase (U/mL)	55.00	56.70 ± 6.70	13.40

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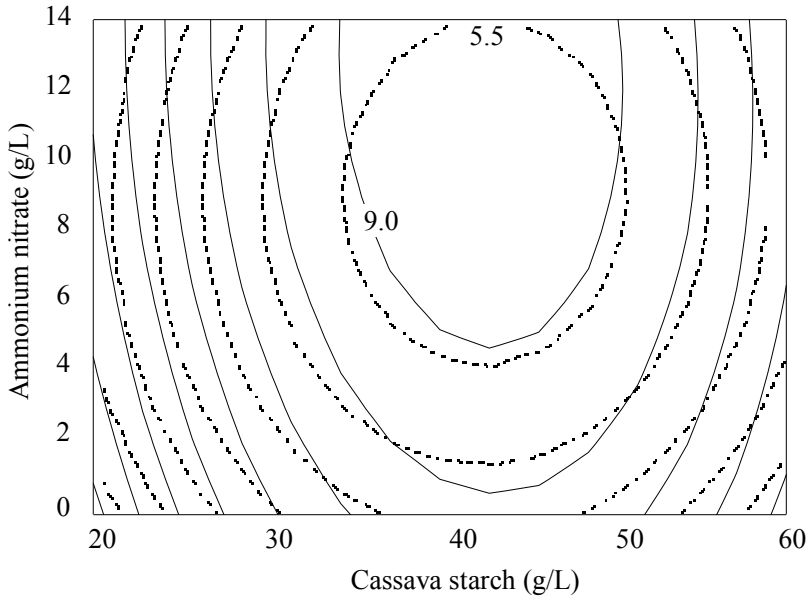

Figure 5. Superimposed contour plots of optimal areas for ABE production (solid line) and butanol production (dashed line). The centre of overlapping area is optimum for ABE and butanol production. The contour lines, 9.0 and 5.5, are the values of ABE and butanol, respectively on the contour lines closest to the centre of the optimal areas.

The low value of CV indicates a close correlation between the experimental and predicted values. The results were also compared to those of the pure culture under the same condition. Time courses of OD_{660}, amylase activity, reducing sugars, acids and ABE production are shown in Figures 6A and 6B for the pure culture and co-culture respectively. The co-culture gave a much faster rate of increase of OD_{660} and amylase activity. The latter reached 56.7 U/mL or about 11.7 times higher than that from the pure culture (4.85 U/mL). Consequently, the co-culture produced a much higher amount of ABE (9.02 g/L), i.e. about 6.9 times more than that obtained from the pure culture (1.3 g/L). Based on the medium optimisation using RSM, ABE production by the co-culture was improved 2.2-fold compared with that obtained using the initial condition in which 20 g/L cassava starch, 5 g/L yeast extract and 2 g/L ammonium nitrate were used in the medium.

The ABE production (9.02 g/L) obtained by RSM optimisation is comparable to the value (9.71 g/L) obtained in the previous study [7], in which the culture condition was conventionally optimised. The amylase activity (56.7 U/mL) from response surface optimisation is also higher than the previous result (49.3 U/mL). The optimum condition determinded by the conventional method requires 40 g/L starch, 32 g/L yeast extract and 2 g/L ammonium nitrate [7], while the optimum condition obtained by RSM requires the same amount of starch, somewhat higher amount of ammonium nitrate (8 g/L), but much lower amount of the costly yeast extract (5 g/L).

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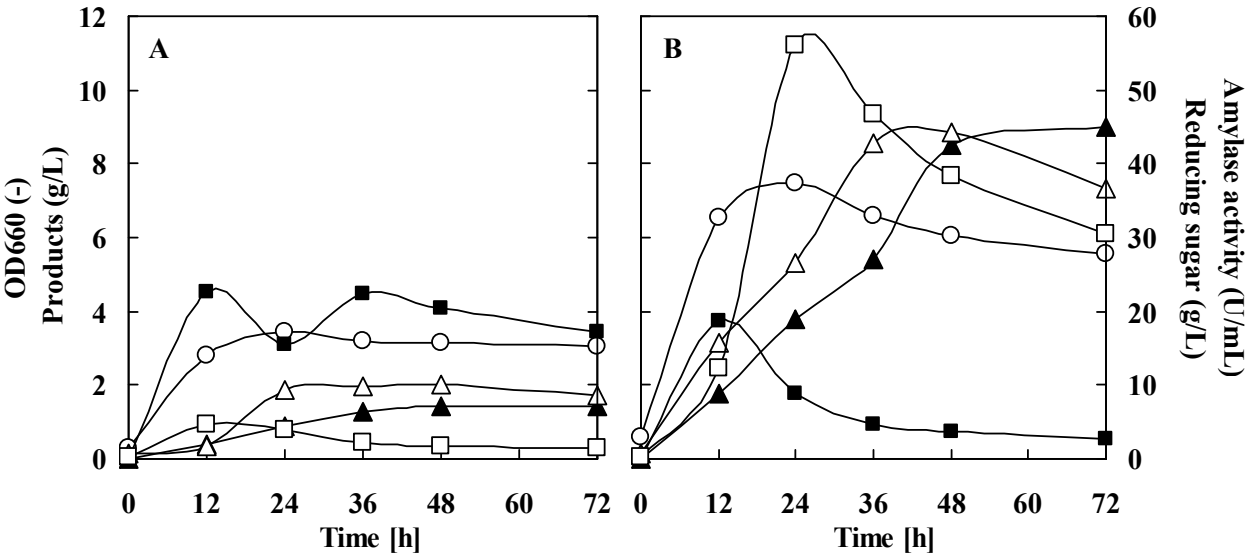

Figure 6. Growth and metabolic activity of pure culture of *C. butylicum* TISTR 1032 (A) and co-culture of *C. butylicum* TISTR 1032 and *B. subtilis* WD 161(B) under optimum condition (40 g/L cassava starch, 5 g/L yeast extract and 8 g/L ammonium nitrate). Legend: OD_{660} - open circle; amylase activity - open square; reducing sugars - filled square; acids (sum of acetic and butyric acids) - open triangle; ABE - filled triangle.

CONCLUSIONS

The optimisation by RSM has shown that starch concentration is the most important factor in ABE production from cassava starch by a co-culture of *Clostridium butylicum* and *Bacillus subtilis* without anaerobic pretreatment. Ammonium nitrate also contributes a significant effect while yeast extract has the least effect. This co-culture system with the cost-effective medium found in this study may contribute greatly to the development of industrialised ABE production.

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