Statistical Optimization of Biobutanol Production from Oil Palm Decanter Cake Hydrolysate by *Clostridium acetobutylicum* ATCC 824

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Oil palm decanter cake (OPDC) is a potential lignocellulosic biomass for the biofuel industry. The fermentation conditions for biobutanol production using glucose from OPDC hydrolysate by *Clostridium acetobutylicum* ATCC 824 were optimized via response surface methodology (RSM). An analysis of variance (ANOVA) using 2–level factorial was successfully screened. Three significant variables were found to influence the biobutanol yield: glucose concentrations in the OPDC hydrolysate, inoculum sizes, and initial pH. The concentration of yeast extract, however, showed an insignificant effect in this study. The batch fermentation was analyzed using central composite design (CCD), and it yielded significant variables and the predicted optimum conditions were 70.00 g/L of OPDC hydrolysate, 16.20% of inoculum size, and an initial pH of 5.20. The predicted yield of biobutanol was 0.09 g/g using 70.00 g/L of glucose. The optimum conditions were validated, and the actual biobutanol yield was 0.11 g/g with 54.86 g/L of glucose consumption. The biobutanol production using synthetic glucose was 15.38% higher when compared to OPDC hydrolysate, but the utilization of OPDC as alternative substrate was still comparable with other findings.

Keywords: Oil palm decanter cake (OPDC); Biobutanol; 2–Level factorial design; Central composite design (CCD)

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INTRODUCTION

The Malaysian Government has embarked on National Biomass Strategy 2020 and 1Malaysia Biomass Alternative Strategy (1MBAS) to develop and accelerate the commercialization of oil palm biomass utilization into biofuel (Agensi Inovasi Malaysia, 2011). The initiative could translate into an incremental of 30 billion Ringgit Malaysia in the domestic gross national income (GNI), an additional 66,000 new jobs, and it could turn the abundance of oil palm biomass into wealth. The Malaysian Government's Economic Transformation Programme (ETP) concluded that lignocellulosic biofuel (bioethanol and biobutanol) is a major economic pillar that can spearhead economic growth by 2020 (Ng *et al.* 2011). In addition, lignocellulosic biofuels are sustainable due to the availability of the biomass and are considered to be environmentally friendly fuels that are free of sulphur and aromatic compounds (Goh *et al.* 2010).

Biobutanol production from lignocellulosic biomass is attracting great interest due to its sustainability and superior characteristics compared to other biofuels derived from cellulosic materials. Biobutanol can be used in current petrol engines without any modifications. It contains a high energy density, less volatile content, and is less corrosive (Dürre 2008). The use of agricultural biomass as a fermentation feedstock could possibly reduce the biobutanol production cost, which is a major problem for the

biofuel industry (Qureshi and Blaschek 2001). Nowadays, butanol is chemically synthesized from petroleum-based material. However, the dependency of butanol production on petroleum is becoming an issue due to the increase in the price, and the extensive consumption of petrol (Wackett 2008). It also leads to an increased carbon dioxide level in the atmosphere, which contributes to the greenhouse effect and global warming.

Thus, a biological process for biobutanol production from biomass has been proposed. However, at present, the biobutanol production through acetone-butanol-ethanol (ABE) fermentation by *Clostridia* faces several challenges. One of them is the complexity of the ABE fermentation due to the biphasic conditions, in which the cells are very sensitive to certain parameters (Wang and Blaschek 2011). Maddox *et al.* (2000) found that the initial pH is very crucial in ABE fermentation in that it controls the metabolic shift from acidogenesis to solventogenesis. During acidogenesis, the acids (acetic and butyric acid) and gases (carbon dioxide and hydrogen) are produced, while solventogenesis produces solvents (acetone, butanol, and ethanol) (Jones and Woods, 1986). In addition, the balance of the concentration of sugars is important to prevent substrate inhibitions that subsequently reduce the biobutanol yield. A very low sugars concentration might reduce the cells growth and interrupt the acidogenesis phase, thus inhibiting the formation of solvents (Ezeji *et al.* 2005). Additionally, ABE fermentation requires a sufficient amount of nitrogen for generating new bacterial cells, where then the carbon to nitrogen ratio becomes an important parameter (Madihah *et al.* 2001; Ibrahim *et al.* 2012). The imbalance value of any of the parameters will inhibit the cells metabolism, thus reducing the production of solvents.

An optimization study for ABE production is very important, especially from cheap biomass, to obtain a high biobutanol yield. The conventional optimization of one or two factors at a time is commonly used to increase the biobutanol yield (Salleh *et al.* 2008; Ibrahim *et al.* 2012). The biobutanol production, however, can be affected by two or more factors simultaneously, and thus a multi-factorial statistical experimental design approach is required. Response surface methodology (RSM) is a statistical approach to evaluate the significant relationship between several independent variables, showing the interactions of the variables that affect the process and determine the optimal conditions for desirable responses (Bezerra *et al.* 2008). This approach has the advantage of reducing the number of experiments required. Such an approach has been shown to work effectively in the bioprocess industry (Vishwanatha *et al.* 2010).

The utilization of sugars from oil palm decanter cake (OPDC) hydrolysate, which was successfully produced in the course of our previous study (Razak *et al.* 2012) for biobutanol production by *Clostridium acetobutylicum* ATCC 824, was further investigated. A 2-level factorial design was used to identify the most significant factors that play a major role in increasing the biobutanol yield. A central composite design (CCD) was applied to determine the optimum conditions of the significant variables, in order to produce a high yield of biobutanol.

MATERIALS AND METHODS

Oil Palm Decanter Cake Hydrolysate Preparation

Oil palm decanter cake (OPDC) was obtained from a three–phase decanter in Alaf Palm Oil Mill, Kota Tinggi, Johor, Malaysia. The production of OPDC hydrolysate was conducted based on the methods developed by Razak *et al.* (2012). The untreated OPDC was soaked in 1% (w/v) of sodium hydroxide and autoclaved at 121°C for 20 min. The pretreated OPDC was filtered and washed with distilled water until no trace amounts of

alkali were detected. In the saccharification process, 50% of crude cellulases cocktail were added to convert the pretreated OPDC into sugars hydrolysate. The crude cellulases cocktail contained a 1:1 ratio of crude cellulase from locally isolated *Trichoderma asperellum* UPM1 (DSM 24606) and *Aspergillus fumigatus* UPM2 (DSM 24607) as described by Razak *et al.* (2012). A phosphate buffer (0.05 M) with a pH of 5.5 was added to control the pH condition. Saccharification was carried out in shaker incubator at 50 °C with agitation of 200 rpm for 72 h. The OPDC hydrolysate containing fermentable sugars was centrifuged at 10,000 rpm for 12 min to remove solid materials. The hydrolysate was concentrated using rotary evaporator at 50 °C for 60 min. The concentrated hydrolysate was filtered through 70 mm micro–filter (Whatman) before being stored at -20 °C prior to ABE fermentation. The analysis of sugars composition using high performance liquid chromatography (HPLC) showed the OPDC hydrolysate contained 98.91 g/L of glucose and 1.02 g/L of maltose.

Bacterial Culture and Fermentation Experiment

Clostridium acetobutylicum ATCC 824 was inoculated in Reinforced Clostridia Medium (RCM) and was heat-shocked in water bath at 80 °C for 2 min. The heat-shocked culture was grown at 37 °C for 48 h in a shaker incubator at 120 rpm. The heat-shocked culture was transferred into fresh RCM media and further grown for 24 h. All the processes were conducted aseptically in anaerobic condition.

In the batch production of biobutanol, the OPDC hydrolysate concentration was adjusted using a phosphate buffer, and the initial pH value was adjusted using 5 M of K_2HPO_4 or KH_2PO_4. Different concentrations of yeast extract were added to the prepared OPDC hydrolysate, and 84 mL of the prepared media was transferred into a 125 mL of serum bottle. The bottle was capped, sealed, and then sparged with nitrogen to remove the oxygen. The prepared media was autoclaved at 121 °C for 10 min. A 2 mL portion of each filter–sterilized P2 solution consisting of buffer solution: KH_2PO_4, 50 g/L; K_2HPO_4, 50 g/L; ammonium acetate, 220 g/L; vitamins solution: para–aminobenzoic acid, 0.1 g/L; thiamin, 0.1 g/L; biotin, 0.001 g/L, minerals solution: $MgSO_4.7H_2O$, 20 g/L; $MnSO_4.H_2O$, 1 g/L; $FeSO_4.7H_2O$, 1 g/L; NaCl, 1 g/L was added to the prepared media (Qureshi and Blaschek 1999; Liu *et al.* 2010). Various inoculums sizes were inoculated into P2 with OPDC hydrolysate medium. Fermentation was carried out at 37 °C in a shaker incubator at 120 rpm for 96 h. A 2 mL of sample was collected for analysis.

Experiment Design and Statistical Analysis

Four variables including the glucose concentrations in the OPDC hydrolysate, inoculum sizes, yeast extract concentrations, and initial pH values were set in a 2-level factorial design as presented in Table 1.

Table 1. 2–Level Factorial Design for Biobutanol Production

Factors	Variables	Unit	Low Level (-1)	Centre Point	High Level (+1)
X_1	Glucose concentrations	(g/L)	20.0	45.0	70.0
X_2	Inoculum sizes	(%)	1.0	10.5	20.0
X_3	Yeast extract concentrations	(g/L)	0.0	2.5	5.0
X_4	Initial pH	–	5.0	6.0	7.0

The design considered the interaction effects among the variables and was used to screen and evaluate the significant variables that affect the response based on the contribution percentage of the tested variables. The design was performed using Design

Expert 7.0.0 (Stat-Ease, Inc., Minneapolis, MN, USA). All experiments were conducted in triplicate and the mean values of the biobutanol yield were recorded as the response. The software was designed with 20 sets of experiments including four centre points. The statistical significance was verified considering a confidence level above 95% or ρ–value less than 0.05.

The significant factors identified in the 2-level factorial experiment were employed in a central composite design (CCD). The half CCD process with three significant variables was designed for 17 experiments conducted in triplicates. The actual and corresponding coded values of three factors (X_1, X_2, and X_3) are given in Table 2.

Table 2. Coded Values for Each Variable of the CCD for Biobutanol Production

Variables		-α	-1	0	+1	+α
X_1	Glucose concentrations (g/L)	30.13	16.55	50.07	70.00	83.59
X_2	Inoculum sizes (%)	1.00	4.85	10.50	16.15	20.00
X_3	Initial pH	5.00	5.20	5.50	5.80	6.00

The statistical and mathematical analyses of CCD were evaluated using Design Expert 7.0.0. The quadratic effects of the significant variables were calculated, as well as their possible interactions on the yield of biobutanol. The significance of each variable was evaluated using analysis of variance (ANOVA). Three-dimension (3D) surface plots were prepared to show the effects of the variables on the response. A quadratic polynomial equation was proposed to describe the mathematical relationship between the variables and the response. The fit of the model was evaluated based on the values of R^2 and the adjusted R^2 coefficient. The predicted optimum conditions were validated by conducting the experiment using the selected optimum values suggested by the model. Statistical significance was evaluated and verified considering the confidence level above 95% or ρ–value less than 0.05. The behaviour of the system was explained by the following second order polynomial equation (Eq. 1) where, Y is the response (biobutanol yield); X_i and X_j are the input variables (glucose concentrations, inoculum sizes, and initial pH) ranging from –1 to +1 which influenced the response variable Y; β_0 is the constant coefficient; β_i is the ith linear coefficient; β_{ii} is the quadratic coefficient, and β_{ij} is the ijth interaction coefficient.

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{j=i+1} \beta_{ij} X_i X_j \quad (1)$$

Analytical Procedures

The reducing sugars were determined using the dinitrosalicylic acid (DNS) method as described by Miller (1959). The glucose concentration was determined using high pressure liquid chromatography (HPLC) equipped with a refractive index detector. Glucose and sugar monomers were separated in a Rezex RPM-Monosaccharide LC-Column with distilled water used as the mobile phase. The column was operated at 75 °C with a flow rate of 0.6 mL/min. Cell concentrations were determined based on an optical density (OD) method using spectrophotometer at 620 nm together with dry cell weight (DCW) analysis. The concentrations of acetone, butanol, ethanol, acetic acid, and butyric acid were analyzed using gas chromatography (GC-17A, Shimadzu, Japan) with a BP20 column equipped with a thermal ionization detector, as described by Ibrahim *et al.* (2012).

RESULTS AND DISCUSSION

Parameter Screening by 2–Level Factorial Design

The significant variables that affected the biobutanol production were screened using a 2-level factorial design. Table 3 shows that the *ñ*–value of the glucose concentrations in the OPDC hydrolysate, the inoculum sizes, and the initial pH were significant at the 95% of confidence level. Wang and Blaschek (2011) optimized biobutanol production by *C. beijerinckii* NCIMB 8052 from maize stalk juice and found that the sugars concentration and initial pH were the highly significant parameters that affected the biobutanol production. The sugars concentration affected cells growth and the pH controlled the metabolic shift from acidogenesis to solventogenesis (Ezeji *et al.* 2005). Many studies have found that at higher initial pH value, the ABE fermentation tends to produce acids rather than solvents (Lee *et al.* 2009; Jones and Woods 1986). The metabolic activity of the cells required suitable value of undissociated acids to transit from acidogenic to solventogenic phase, while at higher concentrations of acids (at mean time refers to acids in the protonated form) may have caused the acid inhibition or "acid crash" phenomenon mentioned by Maddox *et al.* (2000).

The significant value of inoculum size presented in this study, interpreted the relation of the cells density in producing higher yield of biobutanol, and the details will be further explained in CCD analysis. Madihah *et al.* (2001) has previously mentioned that higher cells density might produce higher biobutanol because the presence of each cell acts as a "factory" in producing the acids and solvents. However, suitable amounts of carbon source and cells are necessary at the point where acids turn into the solvents production.

Table 3. ANOVA of Yield of Biobutanol for 2-Level Factorial Design

Source	Sum of Squares	Mean Square	F–Value	ρ–Value
Model	0.031	5.183×10^{-3}	73.86	< 0.0001
(X_1) Glucose concentration	2.761×10^{-3}	2.761×10^{-3}	39.35	< 0.0001
(X_2) Inoculum size	8.403×10^{-4}	8.403×10^{-4}	11.98	0.0047
(X_3) Nitrogen concentration	1.966×10^{-4}	1.966×10^{-4}	2.80	0.1200
(X_4) Initial pH	0.024	0.024	338.86	< 0.0001
X_1X_3	9.479×10^{-4}	9.496×10^{-4}	13.53	0.0032
X_1X_4	2.572×10^{-3}	2.572×10^{-3}	36.66	< 0.0001
Curvature	9.496×10^{-4}	9.479×10^{-4}	13.51	0.0032
Residual	8.403×10^{-3}	7.016×10^{-5}	–	–
Lack of fit	2.512×10^{-4}	2.512×10^{-4}	4.68	0.0534
Pure error	5.907×10^{-4}	5.370×10^{-5}	–	–

Note: $R^2 = 0.9736$, adjusted $R^2 = 0.9605$

The *ñ*–value for yeast extract concentration was 0.12, which was greater than 0.05 and thus considered as insignificant. On the other hand, Kong *et al.* (2004) and Lin *et al.* (2011) showed that the concentration of yeast extract was a significant parameter that affected biobutanol production. The *C. acetobutylicum* ATCC 824 was able to consume a wide range of yeast extract concentrations to produce a high yield of biobutanol. This situation resulted due to the fact that this study aimed for high biobutanol yield (refers to butanol concentration per sugars consumption) instead of the biobutanol concentration in the fermentation broth. Similar findings by Madihah *et al.* (2001) and Ibrahim *et al.* (2012) also showed that the production of biobutanol decreased when higher yeast extract concentration was used. The yeast extract stimulated the growth of the cells, resulting in extensive sugar consumption, but there was a reduction in the cells' tendency to produce the solvents.

From the model analysis, the *ñ*–value of lack of fit was not significant (*ñ* = 0.0534) and the regression model was strongly significant (*ñ* < 0.0001, R^2 = 0.9736). The design results also made it possible to conclude that the second-order model was fitted to the data as shown in Eq. 2.

$$\text{Biobutanol yield (Y)} = 0.14 + 3.26 \times 10^{-3} X_1 - 7.63 \times 10^{-4} X_2 - 6.95 \times 10^{-3} X_3 - 0.02 X_4 + 1.23 \times 10^{-4} X_1 X_3 - 5.07 \times 10^{-4} X_1 X_4 \quad (2)$$

Central Composite Design for Optimization of Biobutanol Production

The CCD was implemented to optimize the significant variables of glucose concentration in the OPDC hydrolysate (X_1), inoculum size (X_2), and initial pH (X_3) based on the results obtained from the 2-level factorial. The CCD data obtained were fitted to a quadratic polynomial and a general second-order model. The statistical significance of the second-order model was analyzed using ANOVA as shown in Table 4. The most significant factors affecting the biobutanol yield were the initial pH values (*ñ* < 0.0001) followed by inoculum sizes (*ñ* = 0.002) and glucose concentrations (*ñ* = 0.0422). Results from the CCD output show that the optimum concentration of glucose, inoculum size, and initial pH were 70 g/L, 16.6%, and 5.2, respectively.

Table 4. ANOVA of Biobutanol Production for Response Surface Quadratic Model

Source	Sum of Squares	Mean Square	F–Value	ρ–Value
Model	0.017	1.85×10^{-3}	227.787	< 0.0001
(X_1) Glucose concentration	5.00×10^{-5}	5.00×10^{-5}	6.151	0.0422
(X_2) Inoculum size	4.21×10^{-4}	4.21×10^{-4}	51.728	0.0002
(X_3) Initial pH	1.25×10^{-3}	1.25×10^{-3}	153.769	< 0.0001
(X_1X_2)	1.19×10^{-4}	1.20×10^{-4}	14.723	0.0064
(X_1X_3)	3.08×10^{-3}	3.08×10^{-3}	379.125	< 0.0001
(X_2X_3)	1.52×10^{-3}	1.52×10^{-3}	186.717	< 0.0001
(X_1^2)	2.29×10^{-3}	2.29×10^{-3}	281.699	< 0.0001
(X_2^2)	1.36×10^{-4}	1.36×10^{-4}	16.688	0.0047
(X_3^2)	1.29×10^{-3}	1.30×10^{-3}	159.274	< 0.0001
Residual	5.69×10^{-5}	8.13×10^{-6}	–	–
Lack of fit	7.74×10^{-6}	7.74×10^{-6}	0.944	0.3687
Pure error	4.92×10^{-5}	8.19×10^{-6}	–	–
Total	0.017	–	–	–

Note: R^2 = 0.996, Adjusted R^2 = 0.992

The three-dimensional (3D) response surfaces with two-dimensional contour plots were plotted as shown in Fig. 1 (A–C). These figures depict the interactions between two variables while keeping the other variables at zero level. The biobutanol yield was set as the response. Figure 1 (A) depicts the interaction of initial pH and glucose concentration in the OPDC hydrolysate. The model predicted that an initial pH of 5.2 and glucose concentration of 70.0 g/L would produce the highest biobutanol yield. The biobutanol production was unfavourable at the low initial pH and low glucose concentration. The variation of initial pH was relatively more important than glucose concentration. At an initial pH below 5 the high acidity interrupted the solventogenic phase due to the deactivation of the enzyme involved in solvents production (Dürre 2008; Ezeji *et al.*

2010). Moreover, the low concentration of carbon source could extend the lag phase and make fermentation unsuccessful (Ezeji *et al.* 2005).

Figure 1 (B) shows the effect of the initial pH and inoculum size on the biobutanol yield. In the interaction curve, the biobutanol yield was very low at an initial pH of 4 and also at a pH of 8. At an initial pH of 5.2 the biobutanol yield increased with an increase in inoculum size up to 16%. Hence, the increase of inoculum size increased the biobutanol yield. The inoculum size affects the time for solvents production from *Clostridium* as studied by Ahn and Balasubramaniam (2007) and Jensen *et al.* (1987). The optimum inoculum size had a direct influence on the growth of bacteria and further led to the highest solvents production. Figure 1 (A) and 1 (B) show that the initial pH is a critical variable that contributes significantly to the biobutanol yield. This study was in agreement with an optimization study conducted by Wang and Blaschek (2011). During acidogenesis, the rapid acids formation decreased the pH and solventogenesis could start at the suitable initial pH; however, the solventogenesis would not proceed under imbalanced pH and poorly buffered medium (Maddox *et al.* 2000).

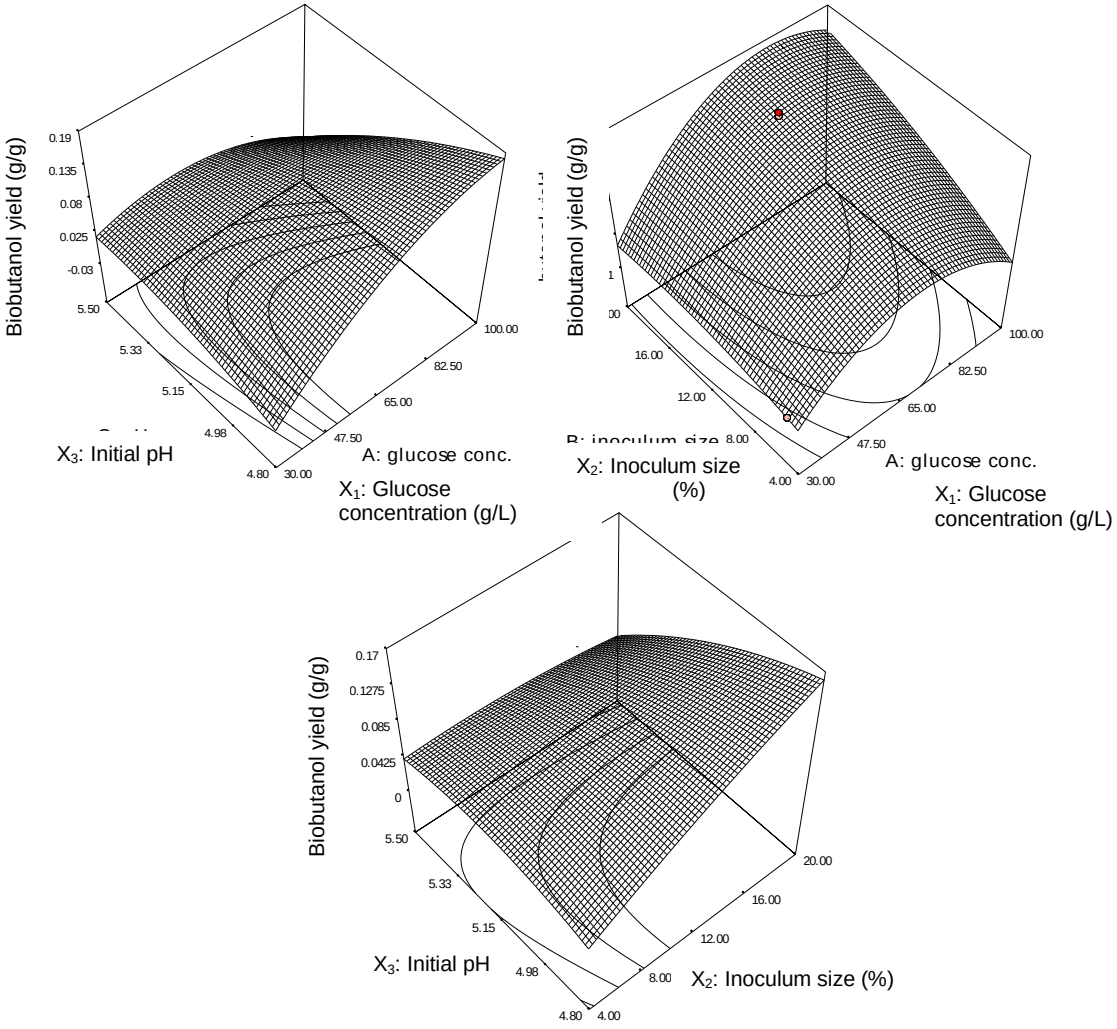

Fig. 1. Response surface and contour plots for biobutanol yield as a result of the interaction between initial pH and glucose concentration (A), the interaction between initial pH and inoculum size (B), and the interaction between inoculum size and glucose concentration (C).

Figure 1 (C) depicts the interaction of inoculum size and glucose concentration in the OPDC hydrolysate. The biobutanol yield increased with an increased glucose concentration and inoculum size. Biobutanol yield started to decrease after a glucose

concentration of 75 g/L was achieved. The greatest biobutanol yield was observed at the curve peak of sugars concentration (70 g/L) and at an inoculum size larger than 16 g/L. Excess carbon in the culture broth reduced the biomass yield and the cells growth due to the metabolism overflow (Dauner *et al.* 2001). The high concentration of substrate with the low inoculum size would prolong the growth phase and inhibit the production of acids. The high inoculum size with low substrate concentration would increase cells competition and reduce the cells growth due to an insufficient supply of carbon.

Validation of Biobutanol Optimization

A validation experiment was carried out to evaluate the conditions predicted by the CCD. The optimized conditions were a glucose concentration of 70 g/L in the OPDC hydrolysate, 16.15% of inoculum size, and initial pH of 5.2. Biobutanol production under the optimized conditions was also compared with production using synthetic glucose as the carbon source (Table 5). The CCD analysis predicted a maximum biobutanol yield of 0.093 g/g and produced biobutanol at a concentration of 6.51 g/L with 70 g/L of glucose consumption. In the actual experiment, the biobutanol yield was 0.11 g/g and the maximum biobutanol concentration was 6.04 g/L with 54.68 g/L of glucose consumption. The actual value obtained had only 7.22% error when compared to the predicted value generated by the model. During the fermentation, an acetone concentration of 3.78 g/L was produced and no ethanol was detected. The total solvents concentration was 9.82 g/L with a 0.18 g/g total solvents yield.

Table 5. ABE Production Using Concentrated OPDC Hydrolysate and Synthetic Glucose by *C. acetobutylicum* ATCC 824

Parameters	OPDC Hydrolysate	Synthetic Glucose
Initial glucose conc. (g/L)	70.00	70.00
Final glucose conc. (g/L)	15.14	7.22
Glucose consumption (g/L)	54.86	62.78
Max. acetone conc. (g/L)	3.78	3.68
Max. bioethanol conc. (g/L)	0.00	1.83
Max. biobutanol conc. (g/L)	6.04	8.17
Max. biobutanol yield (g/g)	0.11	0.13
Total ABE conc. (g/L)	9.82	`13.68
Max. acetic acid conc. (g/L)	4.99	2.84
Max. butyric acid conc. (g/L)	2.16	1.56
Total acids conc. (g/L)	7.15	4.40

In comparison with fermentation using synthetic glucose, *C. acetobutylicum* ATCC 824 consumed a higher amount of synthetic glucose than glucose in OPDC hydrolysate. Lower acids concentration was measured in the final biobutanol using synthetic glucose compared to OPDC hydrolysate. This was due to the fact that the conversion of the acids into solvents in synthetic glucose was higher than the acids conversion in OPDC hydrolysate. Lee *et al.* (2009) also found that the pretreated rice bran hydrolysate produced lower solvents compared to synthetic glucose. The presence of inhibitors released during the chemical and mechanical pretreatment process might be the reason, which reduced the cells growth and glucose consumption (Qureshi *et al.* 2008; Ebener *et al.* 2003). Common inhibitors include furfural, 5-hydroxymethyl-furfural (HMF), and volatile products. The synthetic glucose was prepared using a pure glucose without any presence of foreign substances including inhibitors and acids. Based on the

dark colour of the hydrolysate (even after the centrifugation and filtration process), the OPDC hydrolysate may have contained the inhibitors. The study also observed a higher biobutanol yield using synthetic glucose (0.13 g/g) compared to OPDC hydrolysate that produced 0.11 g/g.

Comparison to Others Substrate and Strains

Biobutanol production from various solventogenic *Clostridia* and different types of biomass hydrolysate from the literature are compared in Table 6. The *Clostridia* species is able to utilize a variety of substrates such as glucose, xylose, and fermentable hydrolysate from lignocellulosic biomass. The biobutanol production in this study was comparable to the studies by Qureshi *et al.* (2008), Lin *et al.* (2011), and Ibrahim *et al*. (2012). Those studies used *C. beijerinckii* BA101, *C. acetobutylicum* CICC 8008, and *C. butyricum* EB6, respectively, with a compliment of various types of biomass hydrolysate as the substrate. The *C. saccharoperbutylacetonicum* N1-4 produced the highest biobutanol concentration in batch fermentation with a concentration of 16.4 g/L and a biobutanol yield of 0.25 g/g; however, they used cassava starch as the substrate (Thang *et al.* 2010).

The pH has been recognized as a key factor in biobutanol production. The optimum pH for producing a high concentration of biobutanol varied depending on the *Clostridia* species, types of substrate, and fermentation conditions. The common initial pH range is between 4 to 7 (Maddox *et al.* 2000; Jones and Wood 1986). However, an initial pH approaching neutrality was identified as not suitable for the solventogenesis process (Maddox *et al.* 2000). The ABE fermentation is considered to be a biphasic fermentation because the involvement of two fermentation phases. During the initial acidogenic phase characterized by rapid growth, acetic and butyric acids were produced, and the culture pH dropped below 5 (usually at 4.8). Then, in the solventogenic phase, the acids were reabsorbed and organic solvents were produced because the medium pH was increased. An inappropriate initial pH causes "acid crash", in which the switch from acidogenic to solventogenic phase ceases (Maddox *et al.* 2000). In the production of biobutanol from OPDC hydrolysate, the optimum initial pH to produce a high concentration of biobutanol was 5.2. This initial pH value was within the range as described in the reported studies.

CONCLUSIONS

A 2-level factorial design was successfully screened, showing that three significant variables (glucose concentrations, inoculum sizes, and initial pH values) influenced the biobutanol yield. An optimization study, using central composite design analysis, suggested that the optimum variables were 70.00 g/L of glucose from OPDC hydrolysate, an inoculum size of 16.20%, and an initial pH value of 5.20 with a predicted biobutanol yield of 0.09 g/g. The suggested variables were validated, and the actual biobutanol yield was 0.11 g/g. The predicted biobutanol concentration was 6.51 g/L while the actual biobutanol concentration was 6.05 g/L. The biobutanol production using synthetic glucose resulted in a 15.38% higher concentration than OPDC hydrolysate, but the utilization of OPDC as alternative substrate for biobutanol production still was comparable to what has been reported in other studies.

Table 6. Comparison of Biobutanol Production by *Clostridia*

Microorganism	Substrate	Initial pH	Biobutanol Concentration (g/L)	Biobutanol Yield (g/g)	References
C. acetobutylicum ATCC 824	70.0 g/L OPDC hydrolysate	5.2	6.04	0.11	This work
C. acetobutylicum ATCC 824	70.0 g/L Glucose	5.5	8.17	0.13	This work
C. acetobutylicum ATCC 824	60.0 g/L Xylose	6.8	7.90	0.13	Sun and Liu (2012)
C. acetobutylicum ATCC 824	50.0 g/L Corn stover hydrolysate	6.5	8.30	0.20	Wang and Chen (2011)
C. acetobutylicum ATCC 824	50.0 g/L Glucose	4.5	12.00	0.25	Li *et al*. (2011)
C. beijerinckii BA101	46.3 g/L Corn fiber hydrolysate	6.8	6.40	0.14	Qureshi *et al*. (2008)
C. acetobutylicum P262	30.0 g/L Sago	6.0	8.40	0.28	Madihah *et al*. (2001)
C. beijerinckii ATCC 55025	53.1 g/L Wheat bran hydrolysate	6.0	8.80	0.24	Liu *et al*. (2010)
C. beijerinckii NCIMB 8052	42.2 g/L Maize stalk juice	6.7	11.50	0.27	Wang and Blashek (2011)
C. butyricum EB6	20.0 g/L oil palm empty fruit bunch hydrolysate	5.5	0.68	0.13	Ibrahim *et al*. (2012)
C. acetobutylicum CICC 8008	44.0 g/L Corn straw hydrolysate	7.0	6.20	0.14	Lin *et al.* (2011)
C. saccharoperbutylacetonicum N1-4	65.9 g/L Glucose	6.0	16.40	0.26	Thang *et al*. (2010)
C. saccharoperbutylacetonicum N1-4	65.1 g/L Cassava chip hydrolysate	6.0	16.20	0.28	Thang *et al*. (2010)

ACKNOWLEDGMENTS

The authors would like to thank AlafPutra Biowealth Sdn Bhd, Research University Grant Scheme (RUGS), and Graduate Research Fellowship (GRF) from Universiti Putra Malaysia for financial support.

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Article submitted: August 10, 2012; Peer review completed: November 1, 2012; Revised version received: November 7, 2012; Accepted: February 10, 2013; Published: February 18, 2013.