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Optimisation of Fermentation Conditions for Isobutanol Production by *Saccharomyces cerevisiae* Using Response Surface Methodology

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Alternative fuels from renewable sources are receiving public and scientific attention due to continuous depletion of petroleum fuel-reserves and environmental problem such as global warming and climate change. Transportation fuel from biofuel is extensively encouraged as it is known to be both renewable and environmentally friendly source of energy. Isobutanol is one of the suitable candidates in replacing gasoline as transportation fuel. This fuel is superior compared to the biofuel that have been commercialised, bioethanol, as it possesses several advantages including high energy content, low solubility, and has lower vapour pressure. Yeast *Saccharomyces cerevisiae* is able to produce small amount of isobutanol naturally as the end product of amino acid degradation. In order to increase the isobutanol production, experiments based on response surface methodology (RSM) with central composite design (CCD) were conducted to study the effects of operating conditions by *Saccharomyces cerevisiae* towards isobutanol yield. The independent variables studied were temperature (28-40 °C), pH (4-7), agitation speed (50-200 rpm), and inoculum size (3-10% v/v). From the experimental results, maximum isobutanol concentration of 200 mg/L was obtained at the optimum condition of temperature (28 °C), pH (7), agitation speed (179 rpm), and inoculum size (10 % v/v). The experimental value (200 mg/L) agreed well with the predicted value from mathematical model (220 mg/L), indicates the suitability of the model and the success of response surface methodology in optimising the operating conditions of isobutanol production. Throughout the study, it can be concluded that the isobutanol yield can be increased by manipulating several factors.

1. Introduction

In recent years, the interest of biofuel (biomass based fuel) production was escalated dramatically in order to replace the petroleum based fuel. Transportation fuel from biofuel is extensively encouraged as it is known to be both renewable and environmentally friendly source of energy (Gonela and Zhang, 2014). The main driving force in promoting the use of biofuel is due to growing environmental concern such as global warming and climate change. Fossil fuel accounted for about 88 % of total energy used in 2008, resulted in large emissions of greenhouse gases (Zhao et al., 2014) as it produces methane (CH_4) and nitrous oxide (N_2O) as well as raises the atmospheric concentration of carbon dioxide (CO_2) (Delavari and Amin, 2014). According to International Energy Agency, about 32 % of CO_2 emission came from transport sector in 2009 (Patrizi et al., 2013). Besides, the depletion of fossil fuel reserves initiated the production of fuel from renewable resources. As fossil fuel is derived from finite sources, the source of this fuel is becoming exhausted. The increasing of global industrialisation and transportation has led to the greater demand of petroleum-based fuels (Ruiz De-La Cruz et al., 2015), consequently increasing the crude oil price thus directly affecting the global economic development and growth. A research from BP (formerly known as British Petroleum) stated that the world's energy

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consumption was increased by 45 % during the past 20 years and is likely to grow by another 39 % in the next 20 years (Patrizi et al., 2013). There are also policies and regulations in producing the biofuel in several countries. In Brazil, renewable sources contributed about half of the total energy supplied in the nation (Pereira et al., 2015).
Biofuel is proven to be non-polluting, sustainable, and reliable thus suitable to be widely used in many areas (Demirbas, 2009). Besides, these fuels can be easily accessed and available in local areas. In particular, the biofuel production is expected to provide benefits such as increase in the energy security and diversity by reducing the dependency on oil imports through finding the new energetic resources, thus directly reducing oil price volatility (Pereira et al., 2015).
Recently, the interest on higher branched chain alcohols (C3-C5) has escalated due to their usage as fuels, fuels substitute, or as chemical derivatives. The increase in carbon atoms leads to the increase in energy density of the alcohol. Higher alcohols have chemical properties similar to that of gasoline thus making them superior compared to bioethanol (Palligarnai et al., 2010). Isobutanol (C4), one of butanol isomer, has been recognised for its potential as fuel additive or substitute due to its attractive physical properties. Some of the attractive behaviours of higher alcohols include higher energy density, lower vapour pressure and less solubility in water. *Saccharomyces cerevisiae* is able to produce isobutanol naturally through fermentation process. The use of natural production host in solvent production offers great advantage, such as the use of heterologous pathway can be avoided (Chen et al., 2011). Nowadays, several strategies have been conducted to increase the isobutanol production in *Saccharomyces cerevisiae* such as expression of genes or by eliminating the competing pathways. The expression of butanol synthesis genes is a complicated process but only low butanol titers are produced. Yeast is well known to be tolerant to alcohols. *Saccharomyces cerevisiae* is able to grow in butanol concentration higher than 20 g/L (Knoshaug and Zhang, 2009). Compared to bacteria, yeast is robust and has high resistance to harsh conditions during fermentation process (Chen et al., 2011). Besides, yeast possesses facultative characteristic so complex facilities such as anaerobic fermentation condition is not required.
Optimisation is a process of improving the performance of a system, process, or a product (Bezerra et al., 2008). Response surface methodology (RSM) is an effective method for optimisation involving multiple variables with minimum number of experiments (Zheng et al., 2008). RSM is a collection of mathematical and statistical techniques based on the fitting of polynomial equation to the experimental data (Bezerra et al., 2008).
This paper is aimed at optimisation of the process variables namely temperature, pH, agitation speed, and inoculum size for high isobutanol concentration. Using RSM with central composite design (CCD), a mathematical correlation between independent variables was determined to get the optimal product yield.

2. Materials and methods

2.1 Inoculum preparation

Baker's yeast *Saccharomyces cerevisiae* was maintained on yeast peptone dextrose (YPD) agar at 4 °C. A full loop of yeast was inoculated into 50 mL flask containing YPD medium (glucose 20 g/L, peptone 10 g/L, and yeast extract 20 g/L) and incubated in a rotating shaker at 170 rpm, 30 °C for 20 h before being used as an inoculum for isobutanol fermentation.

2.2 Microbial fermentation

Batch fermentation experiments were carried out in 250 mL Erlenmayer flask containing 100 mL of synthetic medium consisted of 140 g/L glucose, 8 g/L yeast extract, 8 g/L peptone, 3 g/L $(NH_4)_2SO_4$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, and 0.05 g/L $FeSO_4.7H_2O$. The medium were sterilised at 121 °C for 15 min before starting the fermentation. The temperature, pH, agitation speed, and inoculum size were varied as listed in Table 2. The samples were incubated up to 48 h. During the fermentation process, the samples were taken and analysed for isobutanol concentration.

2.3 Experimental designs and statistical analysis

A central composite design (CCD) of response surface methodology was chosen to explain the combination effects of temperature (°C), pH, agitation speed (rpm), and inoculum size (% v/v) towards isobutanol production. A total of 30 experiments including 6 replications of centre point were designed to optimise the fermentation conditions (Table 2). The relationship between independent variables and response conforms the following polynomial equation as shown in Eq(1).

$$Y = X_0 + X_1A + X_2B + X_3C + X_4D + X_{11}A^2 + X_{22}B^2 + X_{33}C^2 + X_{44}D^2 + X_{12}AB + X_{13}AC + X_{14}AD + X_{23}BC + X_{24}BD + X_{34}CD \quad (1)$$

where Y is the yield of isobutanol (mg/L), X_0, X_1,X_2,…X_{34} represent the estimated regression coefficients, X_1, X_2, X_3, X_4 represent the regression coefficients for linear effects, X_{11} X_{22}, X_{33}, X_{44} are regression coefficients for quadratic effects, X_{12}, X_{13}, X_{14}, X_{23}, X_{24}, X_{34} are regression coefficients of interaction variables, and A, B, C, and D represent the coded experimental levels of the variables. In this study, A, B, C, and D are defined as temperature, pH, agitation and inoculum size, respectively. R^2 and the adjusted R^2 were used as a basis in analysing the fit of the second equation model. In addition, the significant coefficient determined by F-value and p-value at the 95% of significance level were also important in determining the significance of the model.

2.4 Analytical methods

The concentration of isobutanol produced was determined using gas chromatography equipped with flame ionisation detector (GC-FID) and DB-WAX capillary column (50 m length, 0.32 mm inside diameter, and 0.5 µm film thickness). The oven temperature ranging from 40 °C to 120 °C was raised at the rate of 15 °C/min and held at that particular temperature for 4 min. Then the temperature was raised up again with a gradient of 45 °C/min until 230 °C and held for 4 min. The injector and detector temperatures were set at 235 °C and 230 °C, respectively. Nitrogen was used as carrier gas with 4.5 psi inlet pressure.

3. Results and discussion

3.1 Optimisation of isobutanol productivity employing central composite experimental design (CCD)

A two level central composite experimental design was employed to determine the individual and interactive effects of four parameters on isobutanol yield. The experimental and predicted yields are shown in Table 1. The following equation (Eq2) shows the coded value of isobutanol production obtained with the significance terms ($p < 0.005$).

$$\text{Isobutanol Concentration} = 115.42 - 80.52*A + 8.98*B + 17.17*C - 1.11*D - 48.57*A^2 + 31.59*B^2 + 21.74*C^2 + 8.76*D^2 - 1.83*A*B - 11.96*A*C - 1.92*A*D + 2.46*B*C + 4.00*B*D + 0.042*C*D \quad (2)$$

The results were analysed using the analysis of variance (ANOVA). Table 2 shows the coefficient of the model and the significance of the coefficient based on ANOVA. The ANOVA result of the quadratic regression model demonstrates that the model is highly significant due to the F-value and a very low probability value ($p < 0.001$), where the p-value of less than 0.005 shows that the model terms is significant. The p-values are important as a tool to check the significance of each of coefficients (Manikandan and Viruthagiri, 2010). The smaller value of p means more significance to the corresponding coefficients. The coefficient of determination, R^2 of 0.9811 indicates that the model is suitable to adequately represent the relationship among the selected variables. According to Mei et al. (2009), the regression model explained the fermentation well if the R^2 value higher than 0.8.

Figure 1 shows the 3-D surface plots representing the regression equation. The interaction between two variables and their optimum value can be easily understood and located using response surface plot (Kim et al., 2008). Based on Figure 1 (a), the interaction between low temperature (28 °C) and high pH (7) values gives the highest concentration of isobutanol. However as the temperature is increased up to 40 °C, the pH value does not affect the isobutanol titers anymore. Temperature is one of the vital aspects that influence the microbial growth and metabolite production. The rate of enzyme production is directly affected by temperature. The enzyme reactions was increased with temperature increment up to the optimal temperature before being denatured (Togarepi et al., 2012). Le Man et al. (2010) stated that 28-40 °C was the best temperature range for *Saccharomyces cerevisiae* growth. In alcohol production, the biochemical reaction of yeast and its metabolic pathways were strongly influenced by temperature (Fleet and Heard, 1993). The alcohol yield was increased at low temperature but as the temperature was increased, the secondary metabolites productions were also increased (Charoenchai et al., 1998). pH is also one of the factors that affect the microbial growth. pH is used in regulating the protein function and transporting the nutrients into cell (Togarepi et al., 2012). At low pH, the microorganisms tended to produce acids instead of alcohol, while at high pH level, the inhibition in alcoholic production occured due to low adenosine triphosphate (ATP) production during metabolic production in *Saccharomyces cerevisiae* (Pena et al., 1972).

Agitation serves the uniformity of aeration in fermentation broth. Agitation is also important for adequate mixing, mass transfer, and heat transfer (Mantzouridou et al., 2002). Agitation can affect the microorganism's morphological, growth, and product formation. Figure 1 (f) shows the isobutanol production increases proportionally with the agitation speed until the optimal speed is reached. Higher agitation speed leads to the cell structure damage thus decreasing the production. The suitable agitation rate will facilitate the mixing of

Table 1: Comparisons between the experimental and predicted values for isobutanol yield

Run	Variable				Isobutanol Concentration (mg/L)	
	A (^{0}C)	B	C (rpm)	D (%)	Experimental Value	Predicted Value
1	28.00	4.00	50.00	3.00	131.00	121.75
2	40.00	4.00	50.00	3.00	0.00	12.13
3	28.00	7.00	50.00	3.00	157.00	150.46
4	40.00	7.00	50.00	3.00	0.00	-6.49
5	28.00	4.00	200.00	3.00	173.33	175.00
6	40.00	4.00	200.00	3.00	26.00	17.55
7	28.00	7.00	200.00	3.00	210.00	213.54
8	40.00	7.00	200.00	3.00	0.00	8.76
9	28.00	4.00	50.00	10.00	127.00	115.28
10	40.00	4.00	50.00	10.00	0.00	-2.01
11	28.00	7.00	50.00	10.00	150.00	159.99
12	40.00	7.00	50.00	10.00	0.00	-4.63
13	28.00	4.00	200.00	10.00	160.67	168.70
14	40.00	4.00	200.00	10.00	0.00	3.58
15	28.00	7.00	200.00	10.00	238.33	223.24
16	40.00	7.00	200.00	10.00	0.00	10.79
17	28.00	5.50	125.00	6.50	128.00	147.37
18	40.00	5.50	125.00	6.50	0.00	-13.66
19	34.00	4.00	125.00	6.50	132.00	138.04
20	34.00	7.00	125.00	6.50	156.33	156.00
21	34.00	5.50	50.00	6.50	58.00	76.52
22	34.00	5.50	200.00	6.50	123.67	110.86
23	34.00	5.50	125.00	3.00	120.67	125.30
24	34.00	5.50	125.00	10.00	122.00	123.08
25	34.00	5.50	125.00	6.50	125.00	115.42
26	34.00	5.50	125.00	6.50	122.00	115.42
27	34.00	5.50	125.00	6.50	125.33	115.42
28	34.00	5.50	125.00	6.50	106.67	115.42
29	34.00	5.50	125.00	6.50	107.67	115.42
30	34.00	5.50	125.00	6.50	123.00	115.42

Table 2: The result of analysis of variance (ANOVA) for isobutanol production

Source	Sum of Squares	Standard Error	F-value	p-value
Model	1.409E+005	-	55.49	<0.0001*
A Temperature	1.167 E+005	3.17	643.39	<0.0001
B pH	1451.89	3.17	8.00	0.0127
C Agitation	5304.50	3.17	29.25	<0.0001
D Inoculum size	22.22	3.17	0.12	0.7312
A^2	6112.27	8.37	33.70	<0.0001
B^2	2586.23	8.37	14.26	0.0018
C^2	1224.06	8.37	6.75	0.0202
D^2	199.01	8.37	1.10	0.3115
AB	2240.37	3.37	12.35	0.0031
AC	2287.95	3.37	12.61	0.0029
AD	58.79	3.37	0.32	0.5776
BC	96.68	3.37	0.53	0.4766
BD	255.92	3.37	1.41	0.2534
CD	0.0028	3.37	1.547E-004	0.9902
Lack of fit	2342.33	-	3.10	0.1121

Note: *p-value <0.005 is significant.

substrate and product, enhancing the substrate accessibility as well as product distribution. According to Toyoda and Ohtaguchi (2008), the increase of inoculum size results in an increase in both final biomass concentration and ethanol production. In this study, inoculum size of 10 % (v/v) microorganism produced the optimum isobutanol yield. This result agrees well with the research by Erten et al. (2006). They stated that the amount of higher alcohol was increased from 232.79 mg/L to 386.39 mg/L with the increased of inoculum size. The results show that the isobutanol production was mostly influenced by temperature followed by pH value, agitation and inoculum size.

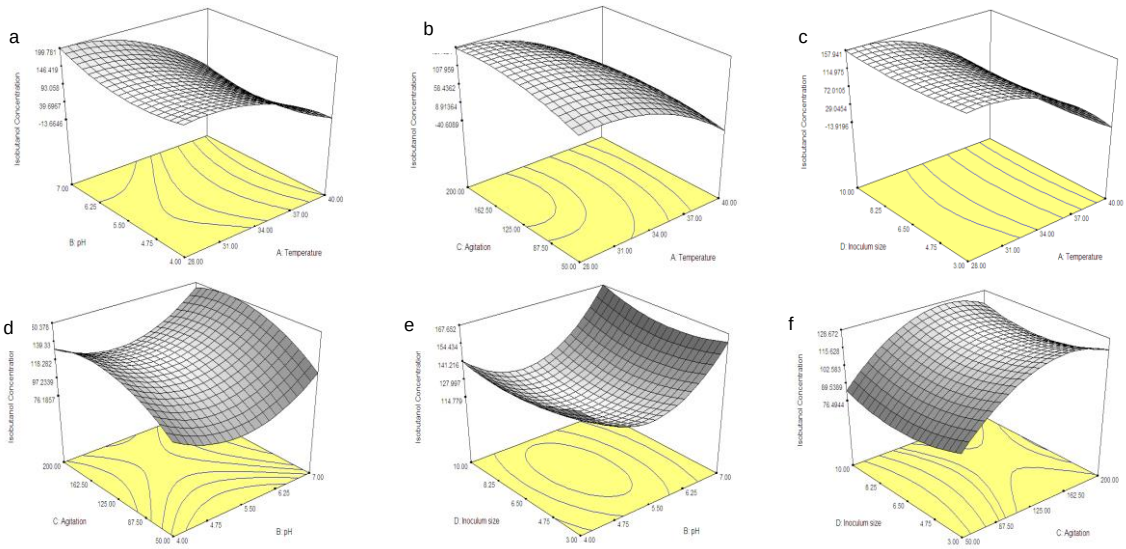

Figure 1: The 3-D response surface plots showing the interaction between the independent variables (a) temperature and pH, (b) temperature and agitation, (c) temperature and inoculum size, (d) pH and agitation, (e) pH and inoculum size, and (f) agitation and inoculum size

3.2 Validation of the model with the optimised fermentation conditions

In order to verify the validity of the optimisation model, confirmation experiments were conducted using the optimised conditions described above. Based on the Design Expert Model, the optimum isobutanol yield of 220 mg/L can be obtained with the optimum condition of temperature (28 °C), pH (7), agitation rate (179 rpm), and inoculum size of 10 % (v/v). The experiments were conducted based on the suggested conditions and the isobutanol concentration produced was 200 mg/L. The agreement between the predicted (220 mg/L) and the experiments (200 mg/L) results verifies the validity of the model.

4. Conclusions

The optimisation of fermentation conditions for the isobutanol production from glucose by *Saccharomyces cerevisiae* was investigated using central composite design (CCD). Isobutanol yield was successfully optimised by RSM with the value of R^2 of 0.9811. A maximum isobutanol yield of 200 mg/L was obtained at the optimum condition of temperature of 28 °C, pH 7, agitation rate of 179 rpm, and inoculum size of 10 % (v/v). Based on the results, the yield of isobutanol can be increased through manipulation of fermentation conditions. Further research focusing on other areas can be done in order to improve the production of biofuels. The increase in isobutanol yield gives a positive impact for biofuel industry. High biofuels production leads to higher consumption of these fuels as transportion fuel, which directly affecting the environmental impact. Besides, the production of biofuel through fermentation process is known to be safe and cause no harm to the environment.

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