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Optimization of Medium Fermentation for Isobutanol Production in *Saccharomyces cerevisiae* using Response Surface Methodology

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Biofuel is known to be a cleaner and sustainable fuel compared to fossil fuels making it more attractive to be used as transportation fuel. Isobutanol, a four-carbon alcohol has a range of physical properties that are more suitable to be used as gasoline substitute than bioethanol. Isobutanol production by *Saccharomyces cerevisiae* is reported in small amount. In order to improve the production of isobutanol, experiments on the optimization of media compositions were conducted in this study. Seven critical nutrients affecting isobutanol production were screened using fractional factorial design. The nutrients involved were glucose ($C_6H_{12}O_6$), ammonium sulfate ($(NH_4)_2SO_4$), yeast extract, peptone, potassium phosphate (KH_2PO_4), magnesium sulphate heptahydrate ($MgSO_4.7H_2O$) and iron sulphate heptahydrate ($FeSO_4.7H_2O$). The screening process shows that glucose, peptone and yeast extract contributed significantly in enhancing the isobutanol yield. The significant factors were further optimized using Central Composite Design (CCD) and Response Surface Methodology (RSM) with the range of glucose (80-140 g/L), peptone (4-8 g/L) and yeast extract (4-8 g/L). From the experimental results,173 mg/L of isobutanol concentration was obtained at the optimum medium compositions of glucose (140 g/L), peptone (8 g/L) and yeast extract (8 g/L). Throughout the study, it can be concluded that isobutanol yield can be maximize via optimization of medium fermentation. With the improvement of technologies nowadays, the isobutanol production is expected to be increased in the future, encouraging the usage of this fuel in the transportation sector worldwide.

1. Introduction

Several concerns such as global warming and climate change (Borawski et al., 2019), depletion of fossil fuel reserves, escalating global energy demand and rising crude oil prices have attracted various attention which leads to the rising interest in liquid biofuel production (Ramli et al., 2017a). Biofuel production from renewable sources is considered as the most sustainable alternative to fossil fuels as it provides positive impact on the environment and the economy (Darda et al., 2019). Currently, the production of isobutanol from biomass as transport fuel attracts public's attention worldwide (Yusoff et al., 2018). Isobutanol has proven to be a better candidate in replacing gasoline as vehicle fuel due to its high energy content (Li et al., 2017), low solubility in water, lower vapor pressure, higher blending ability with gasoline as well as reducing the requirements to modify the current combustion engine (Wechgama et al., 2017).

Saccharomyces cerevisiae produces isobutanol naturally as a by-product of Ehrlich pathway in fermentation with substrate. However, the quantity of isobutanol yield produced is relatively in small amount leading to various alternatives taken by researchers in an effort to increase the yield of isobutanol. Several strategies have been conducted by the researchers to improve isobutanol production yield in fermentation. Most alternatives to enhance isobutanol titers involve modification of microbial genetic such as overexpression of related genes (Song et al., 2020), re-localization of the pathway in the same compartment and deleting the genes that inhibited the product formation (Wess et al., 2019). However, genetic modification is a complex process and several experiments using this approach is unsuccessful in increasing isobutanol yield (Hammer and Avalos, 2017). Optimization is another strategy utilized in obtaining high alcohol yield during fermentation.

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It is expected for isobutanol yield by wild type yeast to be improved through the optimization of fermentation medium compositions. Optimization is a process to discover the best conditions to be applied in the procedure for the optimum response. Response Surface Methodology (RSM) is a method for evaluating the significance of several independent variables, understanding the interactions of various parameters affecting the process and determining the optimal conditions for desirable response (Wang and Blaschek, 2011). The advantage of RSM is reducing the number of experiments required making it easy for overall data analysis.

Medium composition plays an important role in the physiological of microorganisms and their fermentation performance (Hahn-Hagerdal et al., 2005). In order to maintain the growth of the cell, the cultivation medium should contain the necessary nutrients including carbon, nitrogen, vitamins, trace elements, growth factors and metabolic precursors (Huang and Tan, 2007). Nitrogen sources including peptone, yeast extract, urea, ammonium ion, free amino acids and many more have the ability to improve the growth of yeast cell as well as increases the alcohol production. Iron supplementation into fermentation medium is also important as it plays the key roles in numerous cellular function and metabolic pathways for almost all microbes (Zheng et al., 2017).

Reports about optimization of isobutanol production using Response Surface Methodology are virtually lacking. Therefore, the present work was aimed at optimization of the fermentation medium for the production of isobutanol by Saccharomyces cerevisiae. In this study, the effect of several medium compositions on isobutanol yield were evaluated by screening using fractional factorial design. Finally, the most significant variables were optimized by Central Composite Design (CCD) accompanied by RSM for the optimal isobutanol yields. The novelty of this study is to optimize the isobutanol yield using RSM. This method is simpler compared to the genetic engineering approach and has been proven to improve the alcohol production.

2. Methodology

2.1 Inoculum preparation

Saccharomyces cerevisiae used was obtained from baker's yeast (Mauri-pan). The yeast from stock culture was grown on YPD agar and incubated for three days at 30 ± 0.5 °C. A loop of yeast cell from pure culture was aerobically inoculated separately into 10 mL YPD medium broth in a 50 mL test tube and incubated for 20 – 24 h at 30 ± 0.5 °C with orbital shaking at 170 rpm.

2.2 Microbial fermentation

Pre-culture of yeast (5 vol%) was inoculated into 250 mL Erlenmeyer flask containing 50 mL medium. The carbon source was sterilized separately at 121 °C and added to the sterilized fermentation medium. The fermentation was cultivated at 30 °C in an incubator shaker with 170 rpm rotational speed for 48 h.

2.3 Screening medium composition using fractional factorial design

The screening process for the medium composition was done using fractional factorial design. Total of seven media constituents, glucose (20-100 g/L), $(NH_4)_2SO_4$ (1-5 g/L), peptone (1-5 g/L), yeast extract (1-5 g/L), KH_2PO_4 (1-5 g/L), $MgSO_4.7H_2O$ (0.5-2.5 g/L) and $FeSO_4.7H_2O$ (0.01-0.05 g/L) were screened; the media components and their concentration were selected according to the literature with a slight modification (Moon et al., 2011). A total of 70 experiments including 6 times replication of center points was designed to screen the fermentation medium. All experimental runs were carried out in triplicate. The statistical analysis of the data was performed using Design Expert software version 6.0.4 (Ramli et al., 2017b).

2.4 Optimization of medium compositions using response surface methodology (RSM)

Based on the result from the screening process, three media components including glucose, peptone and yeast extract showed positive significant effect towards isobutanol production. These media components were employed for optimization experiment using CCD and RSM. The new concentration of significant medium were glucose (80-140 g/L), peptone (4-8 g/L) and yeast extract (4-8 g/L) while the concentration of $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$ were fixed at an amount of 3 g/L,1 g/L, 0.5 g/L and 0.05 g/L. There are changes in the low and high actual value in this optimization process as this range changed according to the result obtained after the screening process. Twenty experiments with 6 times replication of center points were designed based on the new concentrations. All experimental runs were carried out in triplicate.

2.5 Validation of the result

In order to verify the validity of the optimization model, a new series of experiment was carried out using the optimized medium compositions which is glucose (140 g/L), peptone (8 g/L), yeast extract (8 g/L), $(NH_4)_2SO_4$ (3 g/L), KH_2PO_4 (1 g/L), $MgSO_4.7H_2O$ (0.5 g/L) and $FeSO_4.7H_2O$ (0.05 g/L) obtained from the design software

with temperature of 30 °C and agitation of 170 rpm. The experimental values obtained were compared to those predicted by the models.

3. Results and discussion

3.1 Screening medium optimization using fractional factorial design

Based on the analysis of variance (ANOVA) in Table 1, the model was found to be significant with the R^2 obtained of 0.997, indicating that 99.7 % of the variability in the response can be explained by the model. High value of R^2 implies that the model fits very well and can predict the responses satisfactorily besides indicating a good correlation between the experimental and the predicted values (Ramli et al., 2017b). According to the statistical analysis data, all variables were significant at the 95 % confidence level with p-values less than 0.05 (Table 1). Eq (1) presented the coded value of isobutanol concentration:

$$Isobutanol\ Concentration = 86.81 + 31.05A - 4.23B + 13.17C + 18.99D - 7.10E - 18.70F - 1.58G + 12.32AC + 20.51AD - 10.32AF - 0.31AG - 1.43BC + 2.73BE + 1.68BF - 4.35CD - 5.30CF - +1.43DE - 4.60DF - 4.60DF - 3.06ACD - 3.06ACF - 4.07ADF - 2.71AFG + 1.77BCE - 2.17BCG + 1.81CDF \quad (1)$$

Where A, B, C, D, E, F, and G are glucose, $(NH_4)_2SO_4$, peptone, yeast extract, KH_2PO_4, $MgSO_4.7H_2O$ and $FeSO_4.7H_2O$. Based on the equation, glucose, peptone and yeast extract gives positive effect on isobutanol production yield. Conversely, the other variables showed a negative significant effect on overall production of isobutanol Three positive significant variables were proceeded for the optimization process with new range of concentrations.

Table 1: The result of analysis of variance (ANOVA) for isobutanol production

Source	Sum of Squares	Mean Square	F-Value	Prob > F
Model	1.751E+005	6,038.41	447.77	<0.0001
A	61,690.14	61,690.14	4,574.51	<0.0001
B	1,147.35	1,147.35	85.08	<0.0001
C	11,095.46	11,095.46	822.76	<0.0001
D	23,090.32	23,090.32	1,712.22	<0.0001
E	3,224.82	3,224.82	239.13	<0.0001
F	22,387.64	22,387.64	1,660.11	<0.0001
G	159.39	159.39	11.82	0.0014
AC	9,710.13	9,710.13	720.04	<0.0001
AD	26,923.89	26,923.89	1,996.49	<0.0001
AF	6,819.46	6,819.46	505.68	<0.0001
BC	131.27	131.27	9.73	0.0034
BE	476.77	476.77	35.35	<0.0001
BF	180.03	180.03	13.35	0.0008
BG	41.18	41.18	3.05	0.0884
CD	1210.34	1,210.34	89.75	<0.0001
CE	31.61	31.61	2.34	0.1338
CF	1,795.64	1,795.64	133.15	<0.0001
DE	130.36	130.36	9.67	0.0035
DF	1,356.45	1,356.45	100.58	<0.0001
FG	51.34	51.34	3.81	0.0583
ACD	600.25	600.25	44.51	<0.0001
ACF	600.25	600.25	44.51	<0.0001
ADF	1,058.85	1,058.85	78.52	<0.0001
AFG	471.32	471.32	34.95	<0.0001
BCE	200.65	200.65	14.88	0.0004
BCG	300.42	300.42	22.28	<0.0001
CDF	210.25	210.25	15.59	0.003
Curvature	221.60	221.60	16.43	0.002
Residual	525.94	13.49	-	-
Lack of Fit	431.72	12.70	0.67	0.7792
Pure Error	94.22	18.84	-	-
Correlation Total	1.759E+005	-	-	-

3.2 Optimization of medium compositions using Response Surface Methodology (RSM)

3.2.1 Mathematical model and statistical analysis

The CCD was implemented to optimize the significant variables of glucose, peptone and yeast extract concentration selected by the fractional factorial design. By applying the quadratic regression analysis, the Eq (2) representing the relationship between the isobutanol concentration and the examined variables derived from the RSM is obtained as followed:

$$Isobutanol\ Concentration = 134.45 + 7.54A + 6.00B + 20.29C + 5.26A^2 - 0.22B^2 - 1.11C^2 + 7.54AB + 3.29AC - 9.13BC \quad (2)$$

Where A, B and C are the coded form for glucose, peptone and yeast extract concentration. The value of R^2 obtained in this study was 0.983. The ANOVA in Table 2 demonstrated that the model was high statistically significant with the F-value of 62.55 and p-value of <0.0001 while the lack of fit obtained was not significant (p-value = 0.7521), suggested that this model accurately represented the experimental data and able to properly illustrate the effect of glucose, peptone and yeast extract concentration on the production of isobutanol by *Saccharomyces cerevisiae*.

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

Source	Sum of Squares	Mean Square	F-Value	Prob > F
Model	8,539.46	948.83	62.55	< 0.0001
A	776.13	776.13	51.17	< 0.0001
B	492.35	492.35	32.46	0.0002
C	5,621.08	5,621.08	370.58	< 0.0001
A^2	398.15	398.15	26.25	0.0004
B^2	0.72	0.72	0.048	0.8317
C^2	17.68	17.68	1.17	0.3056
AB	455.11	455.11	30.00	0.0003
AC	86.59	86.59	5.71	0.0380
BC	666.13	666.13	43.92	< 0.0001
Residual	151.68	15.17	-	-
Lack of Fit	52.18	10.44	0.52	0.7521
Pure Error	99.50	19.90	-	-
Correlation Total	8691.14	-	-	-

*Prob> F is equal to p- value

3.2.2 Response surface plot

Optimum medium composition for isobutanol production was obtained using the Response Surface Methodology. Response surface plot of each interaction between the independent variables are illustrated in Figure 1(a-c). The best way in expressing the relationship between the independent variable and the response yield are by developing the response surface plots and contour plots of the equation obtained. Figure 1 (a) shows the interaction between glucose and peptone concentration during constant yeast extract concentration (6 g/L). It can be seen that the isobutanol production increased with the addition of glucose concentration and the maximum yield obtained during the combination of highest glucose and peptone concentration. Figure 1 (b) displays the response surface plot between glucose concentration and yeast extract concentration with constant peptone concentration at 6 g/L. Similar pattern can also be observed in this figure in which the highest concentration of both glucose and yeast extract produced the optimum isobutanol titers.

Glucose, a type of carbon source that is known to be the most crucial nutrients in microorganisms as it serves as the fundamental building block for living cells and as an energy source. *Saccharomyces cerevisiae* is able to ferment glucose, sucrose and fructose without any difficulties with the presence of small quantities of niacin, inorganic phosphorus and magnesium (Kampen, 2014).The budding yeast *Saccharomyces cerevisiae* prefer glucose as the energy source, due to the organism's fermentative lifestyle dictated by the glucose regulation of cellular function (Kim et al., 2013), however the *Saccharomyces cerevisiae* cells are able to use the other carbon sources as well such as sucrose and fructose. Al-Shorgani *et al.* (2013) studied the optimization of medium composition for butanol production by *Clostridium* YM1. The result agrees well with the present studies, which showed that glucose gave the greatest positive effect toward butanol production. In addition, the studies regarding the optimization of butanol concentration using *Clostridium beijerinckii* reported by reference (Singh et al., 2016) also supported the previous research whereby glucose significantly affected the

production of butanol yields. The concentration of butanol improved as the total glucose consumption increased.

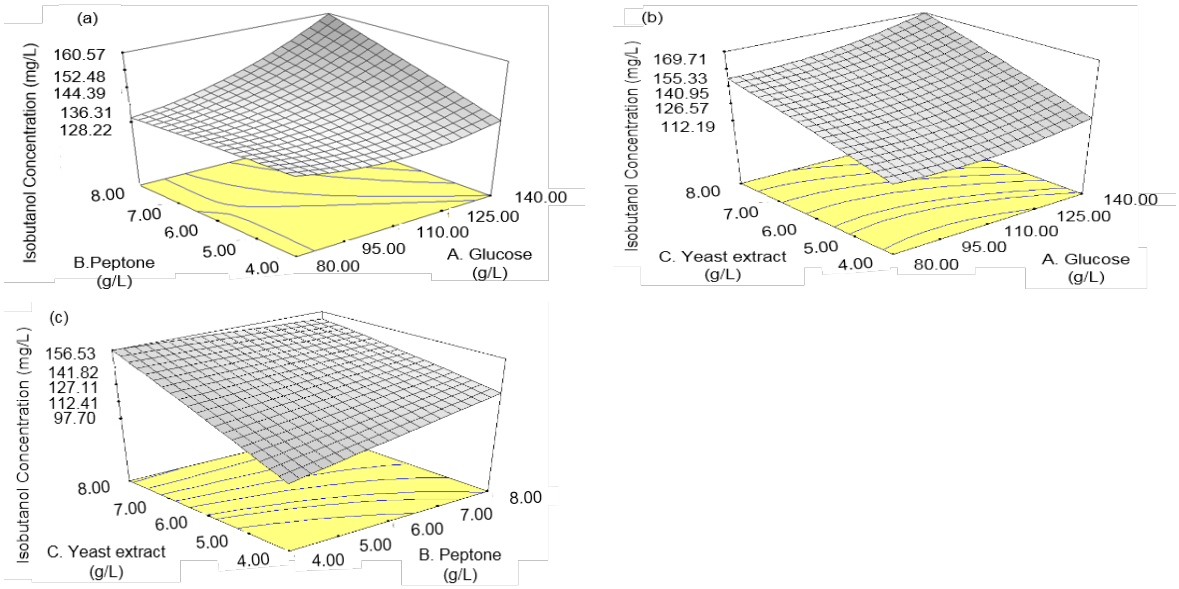

Figure 1: The 3-D response surface plots showing the interaction between (a) glucose and peptone, (b) glucose and yeast extract and (c) peptone and yeast extract

Nitrogen source is generally the next most plentiful components in the fermentation medium after the carbon source (Kampen, 2014). Nitrogen can also serve as an energy source in certain microorganisms. *Saccharomyces cerevisiae* can use varieties of nitrogen sources including organic nitrogen, inorganic nitrogen as well as the combination of both (Li et al., 2017). Peptone is known to be one of the key additives for the growth of fungi; provides amino acids and peptides which are important nutrients for the growth. Yeast extract is also proven to be the efficient nutrient supplementation in alcohol fermentation. This organic substance is primarily consists of a wide range of amino acids, peptides, nucleotides, vitamins, inorganic salts and other soluble component of yeast cells (Li et al., 2017). The three-dimensional response surface plot of peptone and yeast extract concentration with constant glucose concentration at 110 mg/L is shown in Figure 1(c). The plot illustrates that isobutanol yield increases proportionally with increasing peptone and yeast extract concentration. Based on Figure 1 (a-c), the response plots achieved the maximum isobutanol yield during the used of highest amount of peptone and yeast extract coupling with the maximum concentration of glucose proving that both of yeast extract and peptone as a good nitrogen source in isobutanol fermentation.

3.3 Validation of the model with the optimized medium compositions

The RSM showed the best set of medium compositions as the following: glucose of 140 g/L, peptone of 8 g/L and yeast extract of 8 g/L with the predicted value of 172 mg/L isobutanol concentration. Verification experiment was conducted to confirm the model adequacy in predicting the maximum isobutanol yield using the optimum medium compositions. The good agreement between the predicted (172 mg/L) and the experimental result (173 mg/L) verifies the validity of the model and the existence of an optimal point.

4. Conclusion

Based on the screening results, it is exposed that all individual variables affect isobutanol yield significantly. However, only three parameters including glucose, peptone and yeast extract gave the positive response on isobutanol yield thus were proceeded for optimization process. The isobutanol yield was successfully optimized by RSM with the R^2 value of 0.983. A maximum isobutanol yield of 173 mg/L was obtained at the optimum medium compositions of glucose of 140 g/L, peptone of 8 g/L and yeast extract of 8 g/L. Based on the results, it can be concluded that the production of isobutanol can be enhanced by manipulating the fermentation medium compositions.

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