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# Optimization of simultaneous saccharification and fermentation in bioethanol production from sugarcane bagasse hydrolyse by *Saccharomyces cerevisiae* BTCC 3 using response surface methodology

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**Abstract.** The response surface method (RSM) was used to investigate the effects of bioethanol fermentation parameters, including sugarcane bagasse concentrations (0-5 % (w/v), enzyme types (cellulase and hemicellulase), enzyme concentrations (0-1 FPU/g biomass), medium pH, and incubation period. Bioethanol production was conducted by simultaneous saccharification and fermentation (SSF) of sugarcane bagasse hydrolyse using *Saccharomyces cerevisiae* BTCC 3. The experiment results showed that the maximum bioethanol production by using the SSF method was the same as RSM prediction. The optimum conditions for bioethanol production were 5% (w/v) of sugarcane bagasse, a mixture of cellulase and hemicellulase at 1 FPU/g of sugarcane bagasse for each enzyme, pH medium 6.0, and fermentation period 72 hours. The bioethanol yield of 2.43 g/L was obtained under these conditions.

## 1. Introduction

Bioethanol is one of the biofuels that mostly used in the transportation sector. The USA and Brazil are the largest producers of bioethanol in the world with 52% and 37% production, respectively [1]. At present, bioethanol for the fuel market is produced from corn in the United States and from sugarcane in Brazil. These food grade raw materials will not be sufficient to meet the increasing demand for bioethanol fuel. In addition, it accommodates greenhouse gases using bioethanol based on flour or sugar is not as high as desired [2]. On the other hand, bioethanol can also be produced from abundant and renewable biomass sources such as organic wastes of agricultural crops, forests, and other crops [3, 4, 5].

For the large-scale biological production of bioethanol, it is desirable to use cheap and abundant substrates. Bioethanol production cost using corn (the main component of starch) or sugarcane (the ingredients in cane juice or molasses) is 40-70% higher than that of raw materials [6, 7]. Bioethanol production cost is cheaper by using lignocellulose waste products from the forestry, agriculture, and industry [5]. Lignocellulose is a complex polymer consisting of three carbohydrates, namely hemicellulose, cellulose and lignin. This polymer is considered an attractive feedstock for the

production of fuel bioethanol since it is available in a large quantity at low cost [8, 9, 10]. In addition, it can reduce competition with food ingredients.

Several reviews have been published with the theme of bioethanol production from lignocellulosic biomass [8, 11, 12]. Lignocellulosic materials from residues of several different plants have been used and converted to bioethanol. Sugarcane bagasse is one of the major lignocellulosic biomass found in large quantities in the tropical countries. The fibrous residue was obtained after extracting the juice from sugarcane (*Saccharum officinarum*) in the sugar production process [13]. Indonesia is one of the producers of sugarcane bagasse [14, 15] as a raw material for bioethanol production.

The bagasse has a strong crystal structure formed of cellulose, hemicellulose, and lignin. The lignocellulosic sugarcane can be hydrolysed into sugars and fermented into bioethanol [16]. During simultaneous saccharification and fermentation (SSF), the sugars released from biomass are immediately broken down into bioethanol by yeast cells to reduce lead inhibition of enzymes and reduce of production costs [17]. During the SSF process, the ethanol content, concentration, and level of the fermentation products are strongly influenced by operating parameters, including solid biomass concentration, enzyme, temperature, and yeast concentration. Surface Response Methodology (RSM) could be used for analysis of bioethanol production using SSF from sugarcane bagasse including of the biometric series process, concentration and production rate. RSM includes three main methods: (1) implementation of a statistically experimental plan to collect data; (2) development of regression model to correlate experiment data; and (3) prediction of the target response variable against the process parameters using a regression model [18].

The purpose of this study was to analyse the regression model to predict the rate of bioethanol production, concentration and production rate during SSF sugarcane bagasse and to use the regression model to optimize the parameters of SSF process operation including the concentration of solid biomass, and the type and concentration of enzymes.

## 2. Materials and Methods

### *2.1. Microorganism and medium*

*Saccharomyces cerevisiae* BTCC 3 was obtained from Biotechnology Culture Collection (BTCC), Research Centre for Biotechnology, Indonesian Institute of Sciences (LIPI). BTCC 3 strain was cultured in 40 mL of Yeast Peptone Glucose (YPD) medium at 30 °C and 150 rpm for 18 hours. The cells were centrifuged for 15 min for 10,000 g at 4 °C and then washed with aquadest. The cells were collected and diluted with 40 mL of aquadest then used as inoculum for the next study.

### *2.2. Pre-treatment of sugarcane bagasse biomass*

Sugarcane bagasse hydrolysate was used as the substrate for bioethanol fermentation. The dried sugarcane bagasse was ground to 80 mesh of size, hydrolysed with 0.5M $H_2SO_4$, and then filtered to separate the filtrate from the biomass. Sugarcane bagasse hydrolysate with 5% moisture content was used as a substrate in this study.

### *2.3. Fermentation of BTCC 3 strain for ethanol production*

SSF of sugarcane bagasse hydrolysate was carried out on a 100 mL Erlenmeyer flask with 12 mL fermentation medium. Fermentation was performed on a shaker incubator at 30 $^{o}$C and 150 rpm. Yeast Nitrogen Base (YNB) without amino acid as much as 6.7 g/medium was used in this fermentation process. Cellulase and hemicellulase enzymes (Sigma-Aldrich) were used for the saccharification process of sugarcane bagasse.

### *2.4. Response surface method*

Response Surface Method (RSM) was used to determine the optimum conditions of SSF of sugarcane bagasse. The experimental design was provided by Design-Expert 10®. The major composite designs used for bioethanol production by BTCC 3 yeast strain was carried out with five variables, namely concentration of sugarcane bagasse, enzyme type, enzyme concentration, pH medium, and incubation

time for the SSF process (**Table 1**). Total experiments on the main composite design used 31 Erlenmeyer flasks.

**Table 1.** The main composite design for the SSF process for ethanol production by using BTCC3 yeast strain.

| Variable | Code | Degree | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Bagasse hydrolysate (%) | A | 0 | 1 | 3 | 5 | | | | |
| Type of enzyme | B | Control (0) | Cellulase (1) | Hemicellulase (2) | Cellulase-Hemicellulase (3) | | | | |
| Enzyme concentration (FPU/g biomass) | C | 0.2 | 0.4 | 0.6 | 0.8 | 1 | | | |
| pH medium | D | 4 | 4.8 | 5.5 | 6 | | | | |
| Time of incubation (hour) | E | 0 | 6 | 12 | 24 | 30 | 36 | 48 | 72 |

## 3. Results and Discussion

Increasing bioethanol production was along with enhancing the biomass concentration, the enzyme concentration, and incubation period. The lignocellulosic biomass consists of cellulose, hemicellulose, and lignin [19]. Sugarcane bagasse could be converted into simple sugar by lignocellulosic enzymes. Furthermore, the sugar was utilized by BTCC 3 yeast strain to produce bioethanol. Based on the type of enzyme used, the highest ethanol yield was produced during fermentation using a mixture of cellulase and hemicellulase. The efficiency of lignocellulose biomass hydrolysis requires a mixture of enzymes due to their complex structure [20].

### *3.1. Ethanol production*

The RSM analysis resulted in the production of bioethanol in the fermentation of sugarcane bagasse with the two-factor interaction (2FI) model equation. This model is used to predict bioethanol production as a function with five variables, namely A: the composition of sugarcane bagasse (%); B: type of enzyme; C: enzyme concentration; D: pH of the medium; and E: incubation time (hour). The equation for predicting bioethanol production was given by the RSM application as follows:

$$\text{Ethanol (g/L)} = 0.13 + 0.14A + 0.12B + 0.098C + 0.15D + 0.13E + 0.17AB + 0.15AC + 0.16AD + 0.15AE + 0.12BC + 0.15BD + 0.10\,BE + 0.16CD + 0.11\,CE + 0.16\,DE$$

Experimental data and ethanol production prediction were given in **Table 2**. The analysis of variance (ANOVA) shows that the value of squared regression ($R^2$) is 0.9282. This value means that 92.82% of bioethanol production by SSF with sugarcane bagasse hydrolysate could be explained by this model. This is evidenced by the parameters of the optimum conditions for bioethanol production from both of the actual experiments and the predictions were similar (**Table 2**), with the result of the experimental ethanol concentration (2.43 g/L) was slightly higher than the predicted value (2.21 g/L).

The result of the analysis using ANOVA shows that the p-value for this experimental model was <0.0001. This value indicates that the model was significant. The F value also shows an established model. If F value exceeds the tabulated value F at the 0.0001 probability level, the null hypothesis (H0) is rejected at the 0.0001 significance level. ANOVA analysis results show a significant level of five parameters and their interaction in ethanol production.

**Table 2**. Optimum conditions for ethanol production with SSF by BTCC3 yeast strain.

| A Biomass concentration (%) | B Type of enzyme | C Concentration of enzyme (FPU) | D pH | E Time of incubation (hour) | Ethanol (g/L) | | Error (%) |
|---|---|---|---|---|---|---|---|
| | | | | | Prediction | Actual | |
| 5 | 3 | 1 | 6 | 72 | 2.21 | 2.43 ± 6.655E-005 | 6.655E-005 |

The fermentation of sugarcane bagasse to produce bioethanol could take place if the biomass can be converted into sugar, such as glucose, xylose, cellobiose, and others. Cellulase enzymes were used for this process. This study used two types of enzymes, namely hemicellulase and cellulase. The ratio of these two enzymes was 1:1. Based on the type of enzyme applied, the highest bioethanol concentration produces during fermentation using a cocktail of the enzymes was around 0.8 g/L. While the fermentation used a single type of enzyme, 0.16-0.46 g/L of bioethanol were produced by BTCC 3 yeast strain (**Figure 1A**). The optimal enzyme concentration was known at 1 FPU/g of biomass for each type with 0.4 g/L of bioethanol was produced (**Figure 1B**). Sugarcane bagasse hydrolysate was hydrolysed around 5% at an optimum concentration of enzymes and 0.4 g/L bioethanol was produced in this condition. At lower concentrations of sugarcane bagasse and the enzyme concentration has to produce bioethanol with a smaller content of 0-0.18 g/L. In addition, with optimum concentrations of enzymes was obtained bioethanol of 0.8 g/L in pH 6.0 of the medium (**Figure 1D** and **1E**). The optimum time of sugarcane bagasse required 72 hours of fermentation using BTCC 3 yeast strain (**Figure 1F**).

Bioethanol yield in this study was less than another study that could produce 38 g/L of bioethanol [21]. Production of bioethanol could be increased if the concentration of sugarcane bagasse, enzyme concentration, and incubation time were increased. Wang *et al.* fermented 7% (w/v) sugarcane bagasse and used 25 FPU/g-glucan [21]. The similar result was reported by Dasgupta *et al.* using *Kluyveromyces* sp. IIPE453 [22]. This strain produced bioethanol at 17.44 g/L in 7% (w/v) of sugarcane bagasse with additional cellulase enzyme. Other studies reported that the saccharification of sugarcane bagasse with a combination of cellulase and hemicellulase enzymes provided a better response to bioethanol production compared with using only one type of enzyme. The reason is sugarcane bagasse has cellulose, hemicellulose and lignin fibres that required several enzymes to hydrolyse the complex structures of lignocellulose [23, 24].

One other important factor in ethanol fermentation is the pH of the medium [25]. The results of this study indicate that the pH of the medium closer to neutral conditions would provide a positive response to ethanol production. Table 2 shows the ANOVA results for bioethanol production with a fairly high F value and a low Prob>F value<0.005. The value assumed that the predicted function equation for bioethanol production could be used [26]. Sugarcane bagasse fermentation to bioethanol production has an optimum pH medium around 6.0. This condition is related to the BTCC 3 yeast strain characters. BTCC 3 yeast has a pH optimum range from 2.5 to 7.0 (unpublished data). *S. cerevisiae* IFST-072011 also has an optimum pH of 5.0-6.0 in bioethanol fermentation [27]. The pH medium also affects the formation of proteins/enzymes in the cells. The enzyme plays an important role in the process of cell metabolism. In this study hemicellulase and cellulase from Sigma Aldrich have a pH optimum of 5.5-6.0. These results indicate that the suitability of optimum pH for yeast strain and optimum conditions of pH becomes important for the fermentation of sugarcane bagasse.

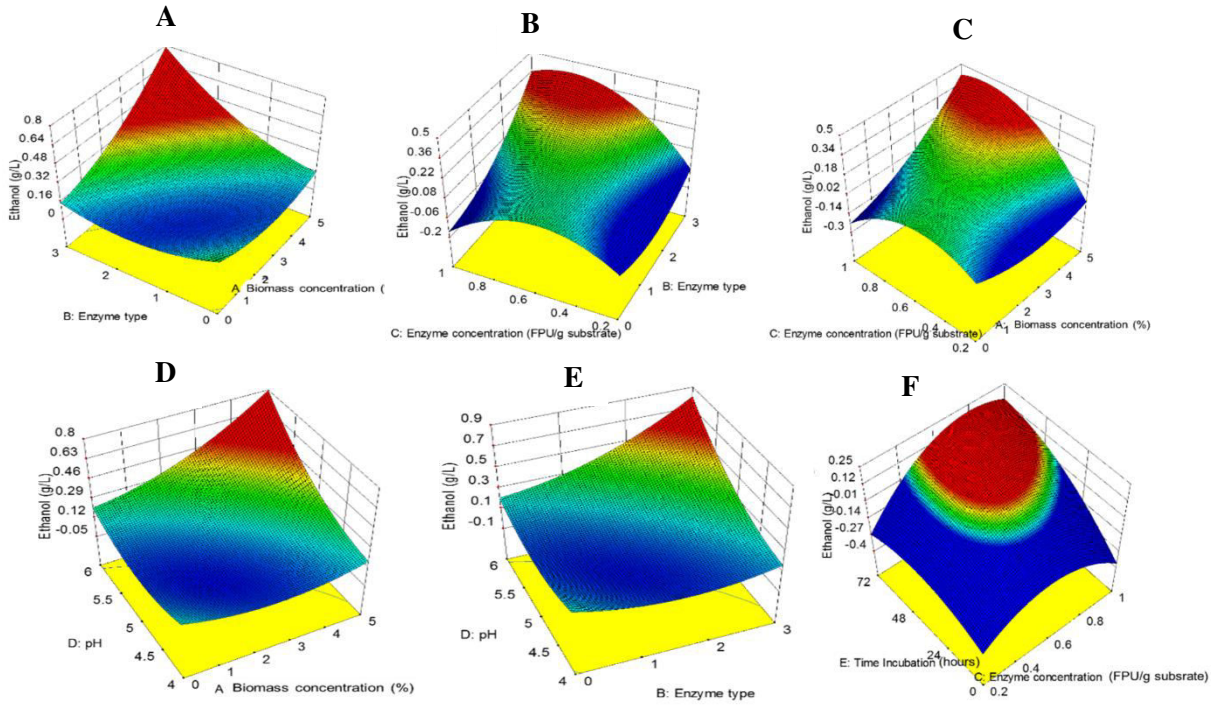


**Figure 1.** The response of ethanol production in several enzyme types (A) and enzyme concentrations (B), bagasse concentrations (C), media pH (D and E), and fermentation time (F). Fermentation took place at a temperature of 30 ° C and agitation speed of 150 rpm.

### *3.2. Glucose*

Based on the ANOVA analysis, the p-value for the experimental model for glucose concentration as a fermentation product was 0.2329. This value indicated the model was insignificant. Significant levels of five parameters and interactions between parameters in glucose concentration were not significant. The application results provide a quadratic model for the glucose concentration as follows:

Glucose (g/L) = 19.06 + 0.29A - 2.65B + 3.01D + 1.03E + 0.67AB + 0.90AC - 4.66AD -2.4AE + 1.15BC + 2.32BD - 0.15BE + 4.06CD-0.77CE - 0.80DE - 11.70A2 + 10.51B2 - 0.12C2 + 0.79D2 - 3.84E2

Description: A: composition of sugarcane bagasse (%); B: type of enzyme; C: enzyme concentration; D: pH of medium; and E: incubation time (hour).

Glucose is one of the sugar types that could be utilized by yeast metabolism to produce bioethanol. Glucose could be produced from hydrolysis of lignocellulosic biomass by using cellulase and hemicellulase. Figure 2 illustrates the higher concentration of sugarcane bagasse did not increase the glucose concentration in the medium. Optimum sugarcane bagasse concentration in producing glucose during SSF process was 3%. This result was assumed the fermentation process in this research used the SSF system. In this fermentation, all saccharification processes and sugar metabolism are performed in one vessel. In this condition, glucose was released as a result of sugarcane bagasse saccharification directly metabolized by BTCC 3 yeast strain to produce bioethanol. Thus, glucose concentration was not detected in the fermentation culture sample. In addition, a high concentration of biomass could inhibit enzyme to hydrolyse substrate. The product of hydrolysis of biomass such as glucose and short chain of cellulose may have inhibited cellulase activity [11].

The ANOVA results for the glucose production model were not significant and the regression values were also low. The incompatibility of this model was reinforced by the optimum conditions between the actual and predictions.

*3.3. Xylose*

The p-value for the experimental model of xylose concentration as a fermentation product from sugarcane bagasse by BTCC 3 yeast strain was <0.0001. This value indicates that the model was significant. The F value also shows an established model. The F value exceeds the tabulated value of F at the 0.0001 probability level, indicates the null hypothesis (H0) was rejected at a significance level of 0.0001.

Experimental data and predictive xylose concentrations are given in Table 3. The ANOVA results show a quadratic regression value ($R^2$) of 0.9711. This value means that 97.11% of xylose concentration formed was influenced by SSF. The parameter of optimum conditions for bioethanol production from the experiments and prediction was similar (**Table 3**). The highest xylose production was 1.44 g/L at 5% sugarcane bagasse, a combination of cellulase and hemicellulase with 1 FPU/g of sugarcane bagasse, medium pH of 6.0, and after 72 hours of incubation period.

**Table 3**. Optimum conditions for xylose concentration with SSF by using BTCC3 yeast strain.

| A Biomass concentration (%) | B Type of enzyme | C Concentration of enzyme (FPU) | D pH | E Time of incubation (hour) | Xylose (g/L) | | Error (%) |
|---|---|---|---|---|---|---|---|
| 5 | 3 (Cellulase + Hemicellulase | 1 | 6 | 72 | Prediction | Actual | 0.000 |
| | | | | | 1.37 | 1.44± 0.000 | |

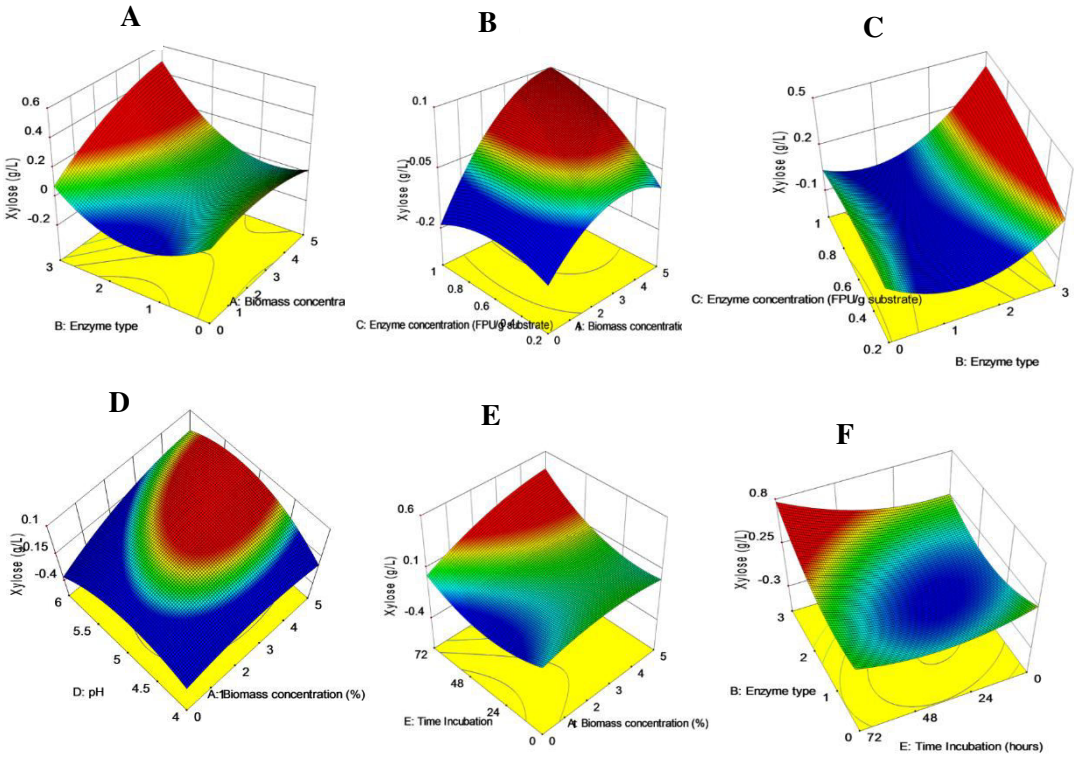


**Figure 2.** Xylose response in enzyme type (A), bagasse concentration (B), enzyme concentration (C), medium pH (D), incubation time and biomass concentration (E), and enzyme type and fermentation time (F). Fermentation was performed at 30 °C and agitation at 150 rpm.

The result of quadratic model application for xylose concentration gives the model as follows:

Xylose = + 0.032 + 0.11A + 0.15B + 0.052C + 0.029D + 0.12E + 0.11AB + 0.051AC + 0.072AD + 0.087AE + 0.076BC + 0.030BD + 0.13BE + 0.10CD + 0.064CE + 0.066DE - 0.069A2 + 0.22B2 -0.036C2 -0.16D2 + 0.15E2

Description: A: composition of bagasse (%); B: type of enzyme; C: enzyme concentration; D: pH medium; and E: incubation time.

Production xylose increased along the enhancement of sugarcane bagasse concentration, enzyme concentration, and time fermentation (**Figure 2**). Hemicellulose sugarcane bagasse was not used in yeast metabolism before hydrolysis it to sugar monomer. ANOVA results for xylose concentration in the medium had a high F-value and low Prob> F value <0.005. Thus, the predicted equation for the xylose concentration in the medium could be used for sugarcane bagasse [26].

## 4. Conclusions

The response surface method (RSM) was used to investigate the effects of bioethanol fermentation parameters, such as sugarcane bagasse concentration, enzyme type, enzyme concentration, medium pH, and fermentation duration. Bioethanol production was used by simultaneous saccharification and fermentation (SSF) of sugarcane bagasse hydrolysate by using *S. cerevisiae* BTCC 3. Maximum bioethanol production using SSF method was the same as RSM prediction. The optimal conditions were 5% (w/v) of sugarcane bagasse, a mixture of cellulase and hemicellulose at 1 FPU/g of sugarcane bagasse for each enzyme, pH 6.0, and 72 hours of fermentation. The bioethanol yield of 2.43 g/L was obtained under these conditions.

## 5. Acknowledgements

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