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OPTIMIZATION OF FERMENTATION TO ENHANCE ETHANOL PRODUCTION FROM PALMYRA SAP(BORASSUS FLABELLIFER) USING SACCHAROMYCES CEREVISIAE

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ABSTRACT

Ethanol is widely needed in various field such as for making food and beverages, pharmaceutical products, personal care and cosmetics, as well as for medical and laboratory purposes. Palmyra is an agricultural product which is abundant in Indonesia, especially in the coastal and tropical area of East Java. Legen is known as traditional drinking juice from fresh palmyra sap. It has high sugar content which reaches 10-15%, that potential for ethanol raw material.In the present study, Palmyra sap has been fermented in a batch-wise fermentor using *Saccharomyces cerevisiae* in a working volume of 100 ml for 80 hours. The optimum independent variables for the fermentation of palmyra sap such as pH, initial inoculum, and sugar concentration have been investigated based on the experimental design by central composite design (CCD) recommendation in response surface methodology (RSM). The result of the experiment at the optimum condition and theoretical prediction obtained yield ethanol of 0.2221 (g/g) and 0.2368 (g/g), respectively.The highest ethanol yield using *Saccharomyces cerevisiae* was 0.2368 (g/g) obtained at pH 4.77, inoculum concentration of 12,740,970 (cell/ml)/(g.l^{-1}), and sugar concentration of 110 g.l^{-1}. The P-value of interaction of variance was <0.005, indicated that the interaction effect was significant.

Keywords: CCD (Central Composite Design), ethanol, palmyra sap (Borassusflabellifer), response surface methodology (RSM), Saccharomyces cerevisiae.

INTRODUCTION

The improvement of ethanol has been increasing developed because it presents as an economical and renewable alternative fuel source. Ethanol also can be used as many purposes, such bulk organic chemical, such as solvent, pharmaceutical and cosmetics, food and beverages.Fermentative ethanol is produced commercially using *Saccharomyces cerevisiae*. Ethanol can be produced by different ways; batch (Widjaja *et al*. 2015), continuous (F. S. Wang *et al*. 2013), fed-batch (Li, Dewan, and Nazmul Karim 2012), and semicontinuous processes (Staniszewski, Kujawski, and Lewandowska 2009). However, most of the ethanol is produced by batch fermentation process because it is economical (Venkata, Bandaru, and Bandaru 2007; Humaidah *et al*. 2017; Widjaja *et al*. 2014, 2015).

Ethanol can be produced from various feedstocks such as from the first-generation from sugar and starch (i.e sugar: sugarcane, sugar beet, sweet sorghum and fruits; and starch) (Bai, Anderson, and Moo-young 2008), second-generation from lignocellulosic biomass (i.e wood, straw and grasses) (Aditiya *et al*. 2016), and third-generation from derivative algal biomass (i.e microalgae and macroalgae)(Phwan *et al*. 2018; Jambo *et al.* 2016). The suitable substrate is the main cost component for industrial ethanol production that essential to be carried out from the cheap substrates. Palmyra is an agricultural product which abundantly raw material based on sugar and it is available in Indonesia, especially in coastal and tropical area of East Java. Legen is known as traditional and most valuable drinking juice from fresh palmyra sap, which can be used as ethanol raw material feedstock due to high sugar content (10 - 15 g/100 ml) (Widjaja *et al*. 2017).

There are many publications about optimization of fermentation efficiency to optimize the ethanol production using yeast and bacteria on the sugar-based material. In this case, *Saccharomyces cerevisiae*,*Pichia stipitis*, and *Zimomonas mobilis* have been reported to be able to produce ethanol by Palmyra palmsap fermentation (Hanh and Kim 2009; Rodhe *et al*. 2012; Chrisnasari *et al.* 2014). *Saccharomyces cerevisiae* is sugar yeast which has been widely used for ethanol fermentation due to its high alcohol tolerance, rapid fermentation rate, and high ethanol yield than other microogranisms (S. Ghosh, Chakraborty, and Raychaudhuri 2012; Widjaja *et al.* 2014, 2015). *Pichia stipitis* is a microorganism that can ferment sugar with no oxygen presence (Staniszewski *et al.* 2009), but it was performed in glucose to xylose as a substrate because rate of glucose is higher than of xylose in the same condition (Widjaja *et al.* 2017). *Z. mobilis* uses Entner Doudoroff (ED) pathway to anaerobically ferment glucose and achieve high ethanol tolerance up to 16 % (v/v) with higher yield of ethanol up to 2.50 times than traditional yeast fermentation, but it produces a low amount of biomass compared with *S. cerevisiae* (X. Wang *et al*. 2018).

The development of the best operating condition for the maximum of ethanol quality and optimum fermentation process is crucial. Therefore, RSM (Response Surface Methodology) as the one of statistical methods that suitable to identify the effect of individual variables and to determine the optimum conditions by combining experimental design with interpolation of first-

second order polynomial equations in a sequential testing procedure, has been chosen.

In this research, the effects of the three variables (pH, amount of inoculum and initial sugar concentration) and their interactions on the optimization of ethanol production were studied. The aim of this study was to know the influence of three affecting factors (pH, amount of inoculum and initial sugar concentration) and their interaction to optimize the ethanol production by *Saccharomyces cerevisiae* in Palmyra sap using factorial design and analysis by Response Surface Methodology

MATERIAL AND METHODS

Chemicals

KH_2PO_4, $(NH_4)_2SO_4$, HCl, NaOH, $MgSO_4.7H_2O$ (Merck), Potato Dextrose Agar (PDA), Nutrient Broth, yeast extract, and DNS reagent were all purchased from Merck. Sodium Potassium Tartrate and Sodium Metabisulfite were purchased from Sigma Aldrich.

Growth culture preparation

Yeast (*Saccharomyces cerevisiae*)were obtained from Industrial Microbiology Laboratory, Chemical Engineering Department, Faculty of Industrial Technology, Institut Teknologi Sepuluh Nopember, Surabaya, Indonesia. *Saccharomyces cerevisiae* were cultured in Potato Dextrose Agar (PDA) medium. For starter preparation, *Saccharomyces cerevisiae* were maintained in medium containing: 130 $g.l^{-1}$ of glucose,1 $g.l^{-1}$ of KH_2PO_4,0.5$g.l^{-1}$of $MgSO_4.7H_2O$, 1 $g.l^{-1}$ of $(NH_4)_2SO_4$, 10$g.l^{-1}$ of yeast extract. The cultures were then incubated at 30 ^oC for 3 days then stored at 4 ^oC in an *incubator* (Unicell).

Fermentation

The fermentation medium was from fresh Palmyra sap, which was transported from Tuban, East Java, Indonesia. The fresh Palmyra sap was filtered and autoclaved at 121˚C and 15 psi for 15 minutes. Fermentation medium was made from the dilution of Palmyra sap using less sugar condition (110, 120, and 130 $g.l^{-1}$). This is due to the range of narrow sugar concentrations, namely 110 and 130 $g.l^{-1}$, respectively. The sugar concentration supposed to be in a larger range because of itsinfluence in ethanol production.

The pH of the medium was adjusted in several variables by adding NaOH or HCl 1 N solution. The medium was inoculated with several different inoculum concentrations based on experimental design. Fermentation was performed in a batch condition using some bottles with 160 ml of volume and 100 ml of working volume. The bottles were incubated in *incubator shaker* at 100 rpm to keep the working temperature at 32^oC. The fermentation runs for 64-80h depending on the glucose concentration left in the broth.

Analytical methods

Measurement of cell growth was estimated using Hemacytometer as described by (References 2015). The concentration of obtained reducing sugar was determined by 3,5-Dinitrosalicylic acid (DNS) method (Miller et al. 1958). The ethanol concentration was analyzed by using Gas Chromatography Scientific GC ULTRA, detector DSQ II, and column MS 220. The observed (Y_{obs}) yield of fermentation were obtained by Eq. (1). Meanwhile predicted (Y*) was described by (Widjaja *et al.* 2017).

$$Y_{obs} = \frac{EtOH\ formed\ from\ fermentation\ (g)}{initial\ sugar(g)} \qquad (1)$$

Experimental design and optimization

Response surface methodology has been widely used for optimization in the field of food science and technology because of a comprehensive theory, high effectiveness, and simplicity. This method is used to determine the optimum operating conditions in a system or to determine the desired operating areas on the factors that affect the response (Kara Ali *et al.* 2017). Sugar concentration (SC, $g.l^{-1}$), inoculum concentration (IC, cell. ml^{-1}/ $g.l^{-1}$), and pH valueswere chosen as the independent variables, and the ethanol yield (Y observed or Y_{obs} and Y prediction or Y^*) were used as the response. The experiment was carried out based on 2^3 factorial Central Composite Design, six axial points ($\alpha=\sqrt{3}$), and five replications at the center point leading to a total number of 19 experiments. The coded level of each factor in the CCD can be seen in Table-1.

Table-1. Independent Variables of Experimental Design for *Saccharomyces cerevisiae*using CCD.

Variable	Symbol	Unit	Coded Factor	
			-1	1
pH	pH	-	4.5	6.5
Inoculum Concentration	IC	$cell.ml^{-1}/gl.^{-1}$	8075000	11500000
Sugar Concentration	SC	$g.l^{-1}$	110	130

The central composite design was the most commonly used in experimental design because it handle same prediction to all points from the center (Satyabrata Ghosh *et al.* 2014). Once the experiments were performed and the model corresponded to the central data on the response of ethanol yield was expressed in second-order polynomial function as below:

$$Y=B_0+\sum_{i=1}^{n} B_i X_i + \sum_{j\leq i}^{n} B_{ij} X_i X_j \quad (2)$$

where, Y is the predicted ethanol yield, subscripts i and j take values from 1 to the number of variables (n), B_0 is the intercept of regression. The $B_i$and B_{ij} values are linear coefficient and quadratic coefficient, respectively. X is the independent variables level.

RESULTS AND DISCUSSIONS

Optimization of fermentation *saccharomyces cerevisiae*

The purposes of this study wereto find the optimum levels of sugar concentrations, inoculum concentration, and pH for ethanol production from Palmyra sap using *Saccharomyces cerevisiae*. Batch process fermentation was conducted using Palmyra sap as the substrate where the variation of pH and inoculum concentrations were affected on the ethanol production.

Results were taken after the fermentation process done, based on the sugar content remained in the substrate. Based on CCD and experimental results, RSM was used to optimize fermentation process design factors (independent variables). The statistical combinations of variables with the predicted and observed responses are presented in Table-2. The regression equation characterizing the influence of different considered variables on process yield was obtained. The second order polynomial equation that was used for predicting ethanol yield is:

$$Y^* = 0.1931 + 0.1826\ SC + 1.4973x10^{-7}\ IC - 0.0215\ pH - 0.0203\ SC^2 + 5.3935x10^{-16}\ IC^2 + 0.0001\ pH^2 - 6.4963x10^{-10}\ SC.IC + 0.0002\ SC.pH - 1.2211x10^{-9} IC.pH \quad (3)$$

Table-2. The Ethanol Yield of Fermentation by *Saccharomyces cerevisiae.*

Run	pH	Sugar Concentration	Inoculum Concentration	Y_{obs}	Y*
1	4.5	110	8075000	0.1360	0.1228
2	6.5	110	8075000	0.0762	0.0754
3	4.5	110	11500000	0.2123	0.2017
4	6.5	110	11500000	0.1399	0.1499
5	4.5	130	8075000	0.1935	0.1688
6	6.5	130	8075000	0.1338	0.1296
7	4.5	130	11500000	0.1780	0.1640
8	6.5	130	11500000	0.1220	0.1204
9	3.77	120	9787500	0.0990	0.1251
10	7.23	120	9787500	0.0585	0.0465
11	5.5	120	6821363	0.1065	0.1212
12	5.5	120	12753637	0.1822	0.1816
13	5.5	102.68	9787500	0.1816	0.1806
14	5.5	137.32	9787500	0.1794	0.1948
15	5.5	120	9787500	0.1237	0.1467
16	5.5	120	9787500	0.1321	0.1467
17	5.5	120	9787500	0.1332	0.1467
18	5.5	120	9787500	0.1945	0.1467
19	5.5	120	9787500	0.1560	0.1467

Table-3. Statistical Significance of Regression Coefficient for Ethanol Production from Palmyra Sap by Saccharomyces cerevisiae.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
SC	1	0.000958	0.000958	0.000958	0.40	0.545
IC	1	0.016320	0.016320	0.016320	6.75	0.029
pH	1	0.028062	0.028062	0.028062	11.60	0.008
SC^2	1	0.010486	0.010486	0.010486	4.33	0.067
IC^2	1	0.000042	0.000126	0.000126	0.05	0.824
pH^2	1	0.030380	0.023450	0.023450	9.69	0.012
SC* IC	1	0.013464	0.013464	0.013464	5.57	0.043
SC* pH	1	0.000130	0.000130	0.000130	0.05	0.822
IC* pH	1	0.000037	0.000037	0.000037	0.02	0.904
Lack-of-fit	5	0.009316	0.009316	0.001863	0.60	0.709
Pure error	4	0.012457	0.012457	0.003114		
Residual error	9	0.021773	0.021773	0.002419		

The statistical significance of regression model is given in Table-3. Itshows that the response of pH, IC, $pH^2$and SC x IC towards P value <0.05 was significant. Meanwhile, SC, IC^2, SC^2, pH x IC, and pH x SC are not significant enough because the P value >0.05. Responses are analyzed using ANOVA (Analysis of Variance) method and model estimation analysis was done by Lack of Fit Test. Lack of Fit is a condition where simple linear regression is not sufficient to match the data (Ghosh, *et al*., 2011). The probability of the prediction model obtained are linear, two factor interaction, and full quadratic. If the P value of the lack of fit model is significant (p <0.05), a more complex model is needed. The P value obtained is 0.707 so that p> 0.05, which indicates that the lack of fit model is not significant in this study. It also implies that the full quadratic experimental model is statistically significant. Table-3 shows the response of the variables of pH, inoculum concentration, pH2, and sugar concentration x the concentration of inoculum was significant with P value <0.05. While sugar concentration, inoculum concentration 2, sugar concentration2, pH x inoculum concentration, and pH x sugar concentration were not significant because P value> 0.05. Most of the above values are significant so the overall model becomes significant.

The relationship between predicted and observed value of yield responses is shown in Figure-1. Linear regression for the predicted and observed yield is shown by the line. The fit of the model gives high R^2 value of 0.82, which directly means that 82% of sample variation in ethanol content is related to the independent variable. This value also indicates that the rest 18% of the variation cannot be explained by the model. It is confidentially said that the regression model is suitable for predicting the optimum value of ethanol because there is only a small difference between the experimental and predictive values (Chrisnasari, *et al*., 2011)

Interaction factors of fermentation palmyra sap usingsaccharomyces cerevisiae

Based on the coefficient of regression, we can determine the sugar concentration, inoculum concentration, pH, and ethanol yield. The optimum yield is 0.2368 (g ethanol/g total sugar) that can be produced at pH 4.8, with inoculum concentration of 12,740,970 cell. ml^{-1}/g.l^{-1}, and sugar concentration of 110 g.l^{-1}.

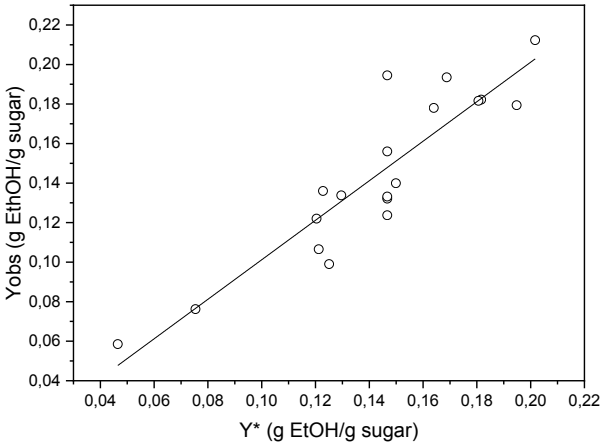
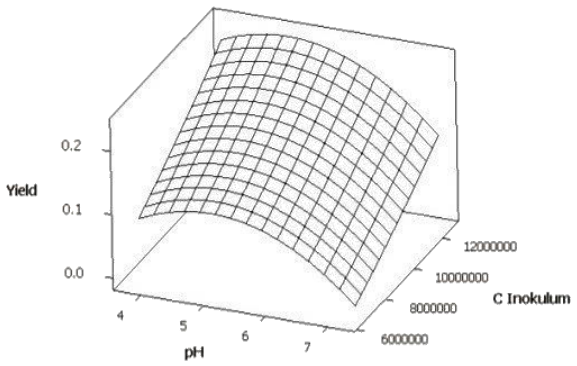


Figure-1. Charts of observed values (Y_{obs}) versuspredicted values (Y*) of ethanol produced from palmyra sap.

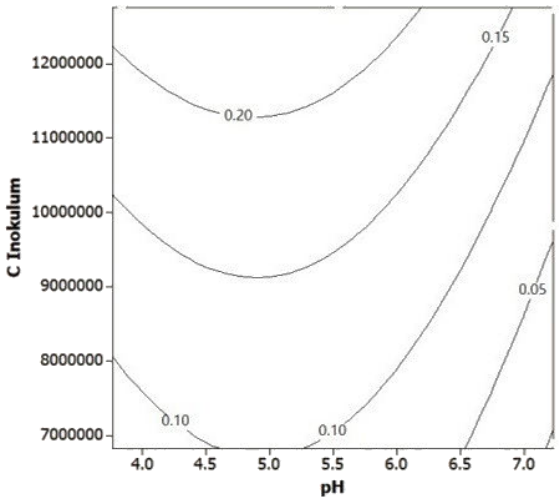
To get an optimum ethanol yield, the range of pH for the fermentation should be around 4-5, and from the experiment, the pH to get the optimum ethanol yield is 4.8, Because butyric acid will be formed if the pH is higher than 5 while Acetic acid production will increase if the pH is lower (Paschapur *et al.* 2009; Kara Ali *et al.* 2017). From Figure-2, the interaction factor between pH x inoculum concentration and pH x sugar concentration has p value >0.05 which means a low influence of

significance. According to Buzes, *et al*. (1998) *Saccharomyces cerevisiae* can grow in a range of pH 3.5 - 5.5, so the pH variation does not significantly affect the growth of microorganisms because the pH range used is still within the range of Saccharomyces cerevisiae growth pH. This can also be seen from the surface of the response graph, where the yield will increase from pH <4 to around pH 5.5 and then will decrease after pH> 5.5 are not included in the pH range for *Saccharomyces cerevisiae* growth. The interaction factor of pH x sugar concentration also did not have a significant effect on ethanol yield because the natural pH range of siwalan juice is 4 - 6 which is also the range of pH used as a variable in this experiment so that it does not have a significant effect on the rate of fermentation, and causes release of synthetic substances and aromatic substances (Humaidah, *et al*., 2017).

Sugar concentration variable was significant over 0.05, because of the narrow range of the sugar concentration (110 $g.l^{-1}$and 130 $g.l^{-1}$). The concentration supposed to be 110 $g.l^{-1}$and 150 $g.l^{-1}$, because the main ingredients itself only contains 139.42 $g.l^{-1}$ sugar, thus the range itself is getting narrowed. Then, the range of the fermentation process is still in the optimum area (Chrisnasari *et al*. 2013). The sugar concentration required to get an optimum ethanol yield is 110 $g.l^{-1}$ because it's the lowest in the coded factor for the experiment. Ethanol was produced when the sugar concentration is relatively low even at anaerobic conditions for *Saccharomyces cerevisiae.* The yeast grows well at sugar concentration lower than 40%, because the higher the sugar concentration, the higher acidity level will be thus will inhibit the rate of fermentation process(Srilekha Yadav *et al.* 2011).



(a)



(b)

Figure-2. (a) Response Surface graph; (b) contour plot for ethanol yield using Saccharomyces cerevisiae.

Figure-2 shows that under the conditions of the same inoculum concentration and varied pH will affect the ethanol production. The optimal pH range for ethanol production through fermentation process is 4-5. *Saccharomyces cerevisiae* can grow in a range of pH 3.5-5.5. This is due to the enzyme activities of the microorganism are affected by pH of the substrate conditions (Satyabrata Ghosh et al. 2014; S. Ghosh, Chakraborty, and Raychaudhuri 2012).

If the amount of inoculum concentration is decreased the amount of ethanol yield will decrease too, and vice versa. In addition, the range of pH and sugar concentrations does not influence the interaction factor any further. From the graph, it is shown that the peak concentration of inoculum and pH is still present in the range of the experimental area, while the response surface peak for ethanol yield is outside the range of experimental areas indicating that ethanol production can be increased more than the yield obtained from the experiments(Chrisnasari *et al.* 2013; Barh and Mazumdar 2008).

After the experiment for optimum fermentation conditions was conducted, the yield of ethanol was 0.2221 (g / g). This value is slightly smaller than the predictive yield of 0.2368 (g / g). The results obtained have a percentage error of 6.2%. This is due to the lack of accuracy in conducting experiments. But the yield value obtained is still higher than the overall yield value of 19 runs of the previous experiment. The type of the contour plot of the optimum point is out of the area of the experiment. From the graph, we can see that the variation of the inoculum variable and pH are taking effect. The optimum point for inoculum concentration and pH are still in the area of the experiment, adversely for ethanol yield which is out if the experiment area. It indicates that the ethanol production still can be increased.

CONCLUSIONS

This present study shows the satisfactory result of RSM design for optimization of the value of pH and initial concentration of inoculum and sugar in the palmyra sap. The highest ethanol yield using *Saccharomyces cerevisiae* was 0.2368 (g/g) obtained at pH 4.77, inoculum concentration of 12,740,970 cell. ml^{-1}/$g.l^{-1}$ glucose, and sugar concentration 110 $g.l^{-1}$

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