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Optimization of Ethanol Production from Palmyra Sap by *Zymomonas mobilis* Using Response Surface Methodology

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Ethanol is believed to be one of the best alternatives to replace gasoline, because ethanol is a renewable energy source and environmentally friendly. The present study focuses on the optimization of palmyra sap as a source for ethanol production. Statistical experimental design using Box-Wilson central composite design was used to optimize the quantitative effects of sugar, urea, and inoculum concentration on ethanol production. It was found that palmyra sap could be used as a substrate for ethanol production using *Zymomonas mobilis* (NRRL B-14234). A maximum ethanol concentration of 58.97 g L^{-1} was obtained after optimizing the parameters of fermentation. The optimum values of sugar, urea, and inoculums concentration were 206.01 g L^{-1}, 3.16 g L^{-1}, and 23.05% (v v^{-1}), respectively, with ethanol yield of 0.3039 g g^{-1}. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to optimize the process for ethanol production.

Key words: ethanol, palmyra sap, *Zymomonas mobilis*, response surface methodology

Etanol merupakan sumber energi alternatif yang berpotensi sebagai pengganti bahan bakar minyak karena bersifat terbarukan dan ramah lingkungan. Penelitian ini bertujuan untuk mengoptimasi nira siwalan sebagai substrat untuk produksi etanol. Pengaruh kuantitatif dari konsentrasi gula, urea, dan inokulum pada produksi etanol dioptimasi menggunakan *response surface methodology Box-Wilson central composite design*. Hasil penelitian menunjukkan nira siwalan dapat dimanfaatkan sebagai substrat untuk produksi etanol menggunakan *Zymomonas mobilis* (NRRL B-14234). Konsentrasi etanol maksimum yang dapat dicapai adalah 58.97 g L^{-1} dengan pengaturan kondisi fermentasi: kadar gula substrat 206.01 g L^{-1}, kadar urea 3.16 g L^{-1}, dan kadar inokulum 23.05% v v^{-1}. Perolehan etanol yang dihasilkan adalah 0.3039 g g^{-1}. Tingginya tingkat kesamaan antara hasil prediksi model dan hasil penelitian aktual merefleksikan akurasi dan kemampuan aplikasi RSM untuk optimasi produksi etanol.

Kata kunci:etanol, nira siwalan, *Zymomonas mobilis*, *response surface methodology*

Due to the progressive depletion of energy resources which mostly based on non-renewable fuels, the era of bioenergy started. Ethanol is believed to be one of the best alternative to replace gasoline because ethanol is a renewable energy source and environmentally friendly (Bai *et al.* 2008). Ethanol can be produced from several different raw materials, such as sugar based (Cazetta *et al.* 2007; Limtong *et al.* 2007), starch based (Jamai *et al.* 2007; Quintero *et al.* 2007) and lignocelluloses based materials (Rogers, 1997; Taherzadeh and Karimi 2007). Selections of appropriate raw materials are important to reduce production cost and to increase the efficiency of ethanol production (Elisson *et al*. 2001).

Palmyra sap (*Borassus flabellifer*) is sugar syrup derived from palmyra tree. The syrup is known to have complete nutrition e.g. sugar, protein, nitrogen, minerals, vitamin B complex that impelled the growth of microorganism (Morton 1988). Palmyra sap is an agricultural product abundantly available in Indonesia, especially in Tuban and the northern coastal area of Java. Palmyra sap is usually used as raw material for jaggery production and for local consumption of fermented products such as toddy, vinegar and traditional alcoholic beverages (Ristiarini *et al.* 2001; Barh and Mazumdar 2008; Sarulli 2009). Jayaseelan and Seevaratnam (1986) used this raw material for ethanol and biomass production using *Saccharomyces cerevisiae* Y18, but there is no report for optimization of ethanol production from this raw material using the other microorganism.

Zymomonas mobilis, a Gram-negative bacterium, have been attracted attention for fuel ethanol production. It is an osmo- and ethanol-tolerant bacterium and has shown higher specific rates of glucose uptake. It was also shown to produce ethanol at rate more than twice the reported rates for yeasts (Rogers *et al.* 1982; Rogers *et al.* 1997; Gunasekaran and Raj, 1999; Jeffries, 2005; Seo *et al.* 2005) via the Entner-Doudoroff pathway under anaerobic conditions. *Z. mobilis* may have a greater potential for industrial ethanol production from raw sugar, molasses, sugarcane juice and sugarcane syrup (Gunasekaran *et al.* 1986; Lee and Huang 2000). Recent process development has demonstrated the

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potential superiority of this organism in other aspects of industrial ethanol production.

To develop a process for maximum production of ethanol, standardization and optimization of fermentation process is crucial. Fermentation process of ethanol needs precise concentration of sugar as carbon source, urea as nitrogen source, and amount of inoculum. Optimization by the classical method using a single dimensional search involving changing one variable while fixing the others at a certain level is laborious and time consuming, especially when the number of variables is large. These drawbacks of single factor optimization process can be eliminated by optimizing all the affecting parameters collectively by Central Composite Design (CCD) using Response Surface Methodology (RSM).

Optimization using RSM is the suitable method for identifying the effect of individual variables and for seeking the optimum conditions for multivariable system efficiently. This method has been successfully applied to optimize fermentation processes (Sunitha *et al.* 1998; Ambati and Ayyanna 2001; Ratnam 2001; Ratnam *et al.* 2003; Ratnam *et al.* 2005; Bandaru *et al.* 2006). A detailed account of this technique has been outlined (Box *et al.* 2005). Basically, this optimization process involves three major steps: performing the statistically designed experiments, estimating the coefficient in a mathematical model and predicting the response and checking the adequacy of the model (Ratnam *et al.* 2005).

In this study, the RSM approach was adopted to locate optimum level of sugar, urea, and inoculum concentration for ethanol fermentation from palmyra sap using *Zymomonas mobilis*, since these parameters play a key role in the enhancement of ethanol yield. Optimization was done with Box-Wilson RSM CCD. Here, we demonstrated the potential of palmyra sap for ethanol production and probably the first report on use of RSM for optimization ethanol production using palmyra sap.

MATERIALS AND METHODS

Bacterial Strain. *Zymomonas mobilis* ZM4 (NRRL B-14234) obtained from ARS Culture Collection National Center for Agricultural Utilization Research, Preoria Il, USA, was used throughout the study.

Growth Medium and Growth Conditions. *Z. mobilis* was maintained on medium having composition (g L^{-1}): glucose, 100; yeast extract, 10; KH_2PO_4, 1; $(NH_4)_2SO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.5 and the cells were grown at a temperature of 35 °C and pH of 5.5.

Production Medium and Fermentation. The fermentation medium was from palmyra sap, which is collected from Tuban, East Java, Indonesia. The fresh palmyra sap was filtered and concentrated by boiling for ± 2-3 h until total sugar concentration ± 450 g L^{-1}. Fermentation medium was made from dilution of concentrated palmyra sap using several sugar concentration (115.9, 150, 200, 250, and 284.1 g L^{-1}). Several different concentrations of urea (1.319, 2, 3, 4, and 4.682 g L^{-1}) were added. The medium was sterilized and inoculated with several different concentrations of inoculum cultures (11.59, 15, 20, 25 and 28.41 % v v^{-1}). Fermentation was carried out in batch condition using waterbath shaker. Fermentation condition was maintained at 30 °C with initial pH 7 and incubated for 60 h.

Analytical Methods. The amount of reducing sugar was estimated using DNS method. The total sugar and total nitrogen concentrations were measured using micro Kjeldahl method. The amount of ethanol was estimated by spectroscopy method (megazyme ethanol kit) at 340 nm. Cell concentration was estimated using spectroscopy at 560 nm (OD_{560}) and the number of cell was counted with haemocytometer.

Experimental Design and Optimization. The aim of this study was to find the optimum levels of sugar, urea and inoculum concentrations for ethanol production from palmyra sap using *Z. mobilis*. Central composite experimental design was used in the optimization of ethanol production. Sugar concentration (X_1, g L^{-1}), urea (X_2, g L^{-1}), and inoculum (X_3, % v v^{-1}) were chosen as independent variables and ethanol concentration (Yi, g L^{-1}) was used as output variable. For statistical calculations the variables Xi were coded as Xi according to Equation (1).

$$x_i = \frac{xi - xi}{\Delta xj}, \quad i = 1,2,3,....k \qquad (1)$$

where, x_i is the dimensionless value of an independent variable, Xi is the real value of an independent variable, xi; is the real value of the independent variable at the center point and Δxj is step change. Table 1 shows independent variable that used in this experimental plan. A 2^3-factorial CCD, with six axial points ($\alpha = \sqrt{3}$) and six replications at the center points ($n_0 = 6$) leading to a total number of 20 experiments was employed in Table 2.

The second degree polynomials (Equation (2)) were calculated with the statistical package(Stat-Ease Inc, Minneapolis, MN, USA) to estimate the response of the dependent variable:

Table 1 Independent variable in the experimental plan

Variable	Coded Level				
	-1,682	-1	0	1	1,682
Total Sugar (g/L), X_1	115.9	150	200	250	284.1
Urea (g/L), X_2	1.318	2	3	4	4.682
Inoculums (% v/v), X_3	11.59	15	20	25	28.41

Table 2 The CCD matrix employed for three independent variables

Experiment Number	X_1	X_2	X_3
1	-1	-1	-1
2	1	-1	1
3	-1	1	1
4	1	1	-1
5	0	0	0
6	0	0	0
7	-1	-1	1
8	1	-1	-1
9	-1	1	-1
10	1	1	1
11	0	0	0
12	0	0	0
13	-1.682	0	0
14	1.682	0	0
15	0	-1.682	0
16	0	1.682	0
17	0	0	-1.682
18	0	0	1.682
19	0	0	0
20	0	0	0

$$Y_i=b_0+b_1X_1+b_2X_2+b_3X_3+b_{11}+b_{22}+b_{33} b_{12}X_1X_2+b_{23}X_2X_3+b_{13}X_1X_3 \quad (2)$$

where *Yi* is the predicted response, X_1, X_2, X_3 are independent variables, b_0, is the offset term, b_1, b_2, b_3 are linear effects, b_{11},b_{22}, b_{33} are squared effects and b_{12}, b_{23}, b_{13} are interaction terms.

RESULTS

RSM is a sequential procedure with an initial objective of leading the experimenter rapidly and efficiently to the general vicinity of the optimum. Since the location of the optimum is unknown prior to running RSM experiments, it makes sense to have a design that provides equal precision of estimation in all directions employed.

The three factors that highly influence the fermentative production are sugar concentration, urea concentration and inoculum concentration. Using CCD, a total number of 20 experiments with different combinations of sugar, urea, inoculum were performed (Tables 1 and 2). The response was taken at the maximum ethanol production which was observed at 60 h. The results were analyzed using the analysis of variance and the estimation model analysis was done using Sequential Model Sum of Squares, Lack of Fit Tests and Model Summary Statistics. Prediction model that might be occur from response surface method were linear, 2FI (two factor interaction), quadratic and cubic. The result showed that suggestion model was Quadratic. The following second order polynomial equation was found to represent the ethanol production adequately:

$$Y_i = -234.38018+1.90689X_1+32.22936X_2+3.72154X_3 -0.057275X_1X_2+0.015335X_1X_3+0.51575\ X_2X_3 -4.93427\text{x}10^{-3}X_1^2-5.27344\ X_2-0.18322X_3^2 \quad (3)$$

Table 3 ANOVA for full quadratic model

Source	Sum of Squares	df	Mean Square	F value	P value Prob > F	
Block	4.59	2	2.30			
Model	3303.77	9	367.09	1325.71	< 0.0001	Significant
A-Sugar	158.16	1	158.16	571.19	< 0.0001	Significant
B-Urea	4.15	1	4.15	14.99	0.0047	Significant
C-Inoculums	346.24	1	346.24	1250.42	< 0.0001	Significant
AB	65.61	1	65.61	236.94	< 0.0001	Significant
AC	117.58	1	117.58	424.64	< 0.0001	Significant
BC	53.20	1	53.20	192.13	< 0.0001	Significant
A^2	2191.23	1	2191.23	7913.53	< 0.0001	Significant
B^2	400.45	1	400.45	1446.22	< 0.0001	Significant
C^2	302.12	1	302.12	1091.10	< 0.0001	Significant
Residual	2.22	8	0.28			
Lack of Fit	1.18	5	0.24	0.69	0.6663	Not significant
Pure error	1.03	3	0.34			
Cor Total	3310.58	19				
Std. Dev.		0.53		R Squared		0.9993
Mean		42.77		Adj R-Squared		0.9986
C.V. %		1.23		Pred R-Squared		0.9944
PRESS		18.46		Adeq Precision		100.278

Table 4 Experimental and the predicted value of ethanol yield

X1	X2	X3	Ethanol (g L^{-1})	
Sugar (g L^{-1})	Urea (g L^{-1})	Inoculums (% v v^{-1})	Actual	Predicted
150	2	15	31.92	31.39
250	2	15	36.11	36.26
150	4	15	30.16	30.86
250	4	15	24.48	24.27
150	2	25	28.43	28.64
250	2	25	49.54	48.84
150	4	25	38.57	38.42
250	4	25	46.64	47.16
115.91	3	20	17.45	17.30
284.09	3	20	28.62	28.75
200	1.32	20	43.42	43.93
200	4.68	20	42.60	42.08
200	3	11.59	36.57	36.49
200	3	28.41	53.37	53.43
200	3	20	58.10	57.92
200	3	20	57.92	57.92
200	3	20	57.01	57.92
200	3	20	58.40	57.92
200	3	20	58.20	57.92
200	3	20	57.89	57.92

The coefficients of the regression model (Eq. 3) calculated are listed in Table 3, in which they contain three linear, three quadratic, three interaction terms and one block term. The effects of all three parameters, i.e. sugar, urea and inoculum and their interactions with each other, on ethanol concentration were found to be significant (P value (Prob>F) $\leq$ 0.05), indicating the model terms are significant).

The corresponding analysis of variance (ANOVA) was also carried out to check the best fit of the model. The F value for the best fit of the model was 1325.71. The F value for lack of fit test was *0.69* means lack of fit of the model was not significant, which was desirable. The coefficient of determination (R^2) was 0.9993, which implied that 99.93% of the sample variation in the ethanol yield is attributed to the independent variables. The R^2 value also indicated that only 0.1% of the variation could not be explained by the model. The value of R is 0.9986.

The parity plot showed a satisfactory correlation between experimental values and predictive values (Fig 1), wherein, the points cluster around the diagonal line which indicates the good fit of the model, since the deviation between the experimental and predictive values was low.

Optimum level for sugar, urea and inoculum concentration could be predicted using the equation model. Fig 4-6 represent the iso-response contour and surface plots for the optimization of fermentation conditions of ethanol production.

An increase in the sugar and urea concentration up to an optimum point increased the ethanol production to a maximum level; however, a further increase in the concentration reversed the trend (Fig 2). The optimum sugar and urea concentration for maximum ethanol production lies near the center point. The interaction effect of the inoculums and sugar concentration on the ethanol production clearly indicates a proper combination for production of ethanol (Fig 3). An increase in the inoculum and sugar concentration increased the ethanol production gradually, but at a higher inoculums and sugar concentration, the trend was reversed. A similar effect on the response was observed for the urea at any level of inoculums concentration. An increase in the urea and inoculums concentration up to an optimum point increased the ethanol production to maximum level, but a further increase in the urea and inoculum concentration decreased the ethanol production (Fig 4).

Therefore, the optimum condition was observed near the central value of sugar, urea and inoculum. The predicted optimum condition for maximum ethanol concentration were 206.01 g L^{-1} sugar, 3.16 g L^{-1} urea, and 23.05% v v^{-1} inoculum concentrations. With these optimized parameters, the maximum ethanol concentration obtained was 58.97 g L^{-1}.

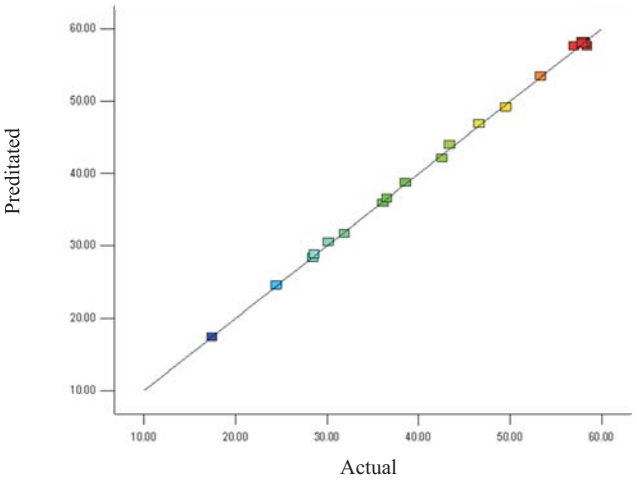

Fig 1 Parity plot showing the distribution of experimental vs. predicted values of ethanol yield.

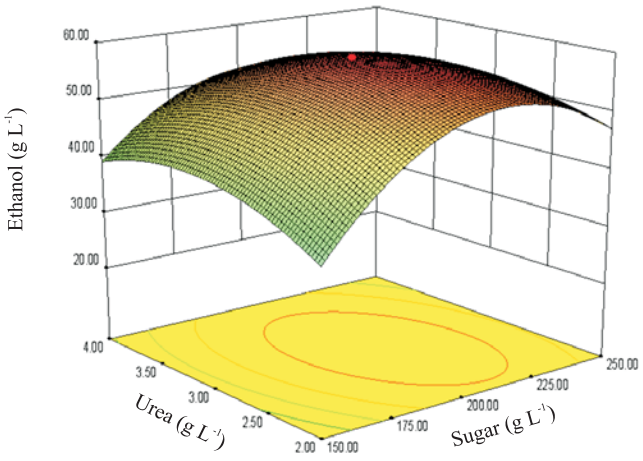

Fig 2 Response surface and contour plot of sugar vs. urea concentration on ethanol production (inoculums was kept constant at 20% v/v/).

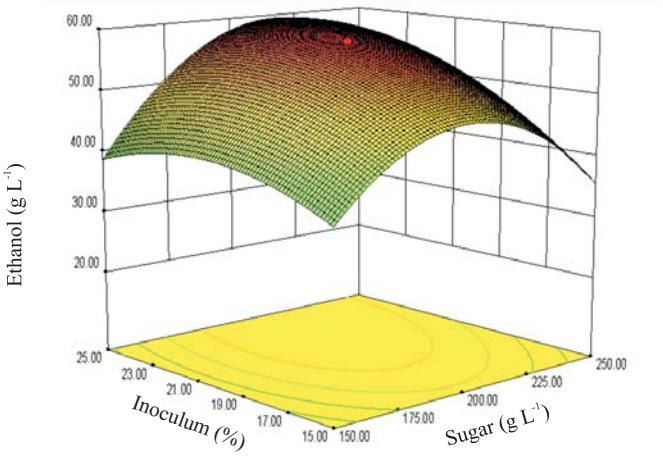

Fig 3 Response surface and contour plot of inoculums vs. sugar concentration on ethanol production (urea was kept constant at 3 g L^{-1}).

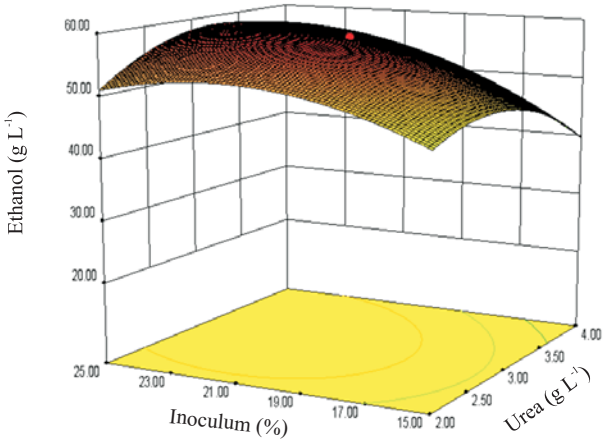

Fig 4 Response surface and contour plot of urea vs. inoculums on ethanol production (sugar concentration was kept constant at 200 g L^{-1}).

DISCUSSION

The experimental and predicted ethanol production at optimum fermentation conditions were also determined (Table 5). The experimental maximum ethanol concentration of 59.77 g L^{-1} was obtained at optimum condition. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of RSM to optimize the process for ethanol production. This result was nearly equal with the data of optimization ethanol production from the others sucrose based material e.g. sucrose, cane juice and molasses (Gunasekaran *et al.* 1986; Lee and Huang 1995; Cazetta *et al.* 2007), because the sucrose content of sugar syrup from palmyra sap was up to 92% of total sugar (Davis and Johnson 1987; Barh and Mazumdar 2008).

The ethanol yield calculated from the experimental data was listed in Table 6. The ethanol yield at optimum condition was 0.3039 g g^{-1}. This obtained result was low because the percent yield of theoretical from this experiment was 59.5% whereas the capability of

Table 5 The of experimental and predicted ethanol yields at optimum condition.

Variables	Optimum Value	Optimum Ethanol Yield (g L^{-1})	
		Experimental	Predicted
Sugar (g L^{-1})	206.01	58.97	59.77
Urea (g L^{-1})	3.16		
Inoculums (%v v^{-1})	23.05		

Table 6 Ethanol yields

Paramaters	Value
Ethanol yield, $Y_{p/s}$ (g/g)	0.3039
Ethanol yield, (% of theoretical)	59.5

theoretical yield of *Zymomonas mobilis* should be 97%. When *Z. mobilis* grows in sucrose, it converts sucrose into glucose and fructose using up to three sucrose-splitting enzymes and produce ethanol, levan and sorbitol (Sprenger 1996). Ethanol production by *Z. mobilis* using sucrose as carbon source is substantially reduced as a result of the levan and sorbitol formation (Lee and Huang 1995). Levan is produced by Z. *mobilis* only when the growth medium contains sucrose, but not when the medium contains a mixture of glucose and fructose (Dawes *et al.* 1996). To solve the problem, invertase can be added to the medium to hydrolyze sucrose. Lee and Huang (1995) reported that when sucrose was previously hydrolyzed by invertase, the level of levansucrase secretion will be lower and the formation of the total levan and sorbitol will decrease, hence increasing the ethanol production.

In other hand, Jayaseelan and Seevaratnam (1986) and Ratnam *et al.* (2005) reported the ethanol production from palmyra sap and jaggery using *Saccharomyces cerevisiae*. Compared with the previous report, it showed that *Saccharomyces cerevisiae* could produce higher ethanol on palmyra sugar than *Zymomonas mobilis.*

Optimization using RSM with Cental Composite Design method enables to find the importance factors at different levels. A high similarity was observed between the predicted and experimental results, reflecting the accuracy and applicability of RSM to optimize ethanol production. The results of this study clearly indicated that RSM is an effective method for maximum production of ethanol from palmyra sap using *Zymomonas mobilis.* The addition of invertase or coupling with the other invertase-producing microorganisms seemed to increase the yield of ethanol production from palmyra sap by *Zymomonas mobilis*.

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