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Comparative Study of Microorganism Effect on The Optimisation of Ethanol Production from Palmyra Sap (Borassus flabellifer) Using Response Surface Methodology

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The energy demand in Indonesia increases due to a significant growth in population, yet fossil fuel storage as the main non-renewable energy source has been significantly depleted. There are a lot of researches on renewable energy; one of the most prominent is the development of bioethanol as a result of fermentation of the sugar. One of the most abundantly available sugar sources in Indonesia is the palmyra sap. Palmyra sap from palmyra tree (Borassus flabelliffer) is a seasonal and low priced drinking juice in tropical countries such as Indonesia and it is highly available in a coastal area of Indonesia. Currently in Indonesia, ethanol is mainly produced from molasses or cassava which can hamper the needs of crop plants. Palmyra sap can be used as a cheap alternative of ethanol feed stock and in the same time can enhance its economic value. Conventional fermentation is usually carried out with the help of Baker's yeast (Saccharomyces cerevisiae), but recent study shows that Gram-negative bacteria Zymomonas mobilis could be a promising alternative of fermentative microorganism. This research aims to determine and compare physical parameters needed in the fermentation of palmyra sap by Saccharomyces cerevisiae and Zymomonas mobilis to obtain optimum concentration of ethanol. Using a batch fermentation process in a 1.8 L bioreactor with 1 L working volume, these microorganisms were cultivated in sterilised palmyra sap and the different physical parameters applied are pH and inoculum concentration. Response surface methodology was generated based on the result of 13 runs generated by central composite design (CCD). Response surface methodology was applied to determine the optimum conditions for the maximum yield of ethanol with the variation of temperature and pH. The highest yield of ethanol concentration using Saccharomyces cerevisiae was obtained at pH 5.05 with inoculum concentration of 3,973,760 cell.mL^{-1}/gL^{-1}glucose. The model showed that the value of R^2 (0.9201) was high and from ANOVA analysis the model is said to be significant. Highest concentration of ethanol obtained by fermentation is 85.68 g/L, with yield of 0.49 and error compared to predicted value is 9.05 %. The highest yield of ethanol concentration using Zymomonas mobilis was obtained at pH 5.57 with inoculum concentration of 2,660,000 cell.mL^{-1}/g.L^{-1}glucose. The model showed that the value of R^2 (0.9290) was high but analysis of ANOVA shows that the model is not significant. Highest concentration of ethanol obtained by fermentation is 12.75 g/L, with yield of 0.49 and error compared to predicted value is 0.65 %. The study concluded that fermentation with Saccharomyces cerevisiae gives better model of optimisation but Zymomonas mobilis gives better result of ethanol production.

1. Introduction

Ethanol is one of the largest volumes of organic chemical industrially produced. Ethanol is believed to be one of the best alternative to replace gasoline because ethanol is a renewable energy source and environmentally friendly. Fermentation is carried out in a batch reactor. Batch fermentation is a widely used process to produce ethanol. Despite its certain disadvantages, such as inhibition by higher sugar content, limited concentration of ethanol yielded (12 %v/v) and low productivity (Widjaja et al., 2016), batch fermentation is still chosen due to higher ethanol yielded compared to other methods. Batch fermentation is able to utilise high population density of microorganism to produce higher ethanol concentration (Bai et al., 2008). The fermentation of

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palmyra sap can be carried out with the help of wine yeast (Saccharomyces cerevisiae). Besides conventionally used wine yeast, Zymomonas mobilis is found to be a promising alternative to produce ethanol from sugar material (Chrisnasari et al., 2011). To develop a process for a maximum production of ethanol, standardisation and optimisation of fermentation process is crucial. In recent studies, optimisation of palmyra sap fermentation to produce ethanol only covers pH, sugar condition, nutrition and temperature. Fermentation process of ethanol needs a precise pH and amount of inoculum. Optimisation 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. These drawbacks of single factor of optimisation process can be eliminated by optimising all the affecting parameters collectively by Central Composite Design (CCD) using Response Surface Methodology (RSM).

2. Materials and method

2.1 Bacterial strain

Saccharomyces cereviciae and Zymomonas mobilis were obtained from Industrial Microbiology Laboratory, Chemical Engineering Department, ITS Surabaya, Indonesia.

2.2 Growth medium and growth condition

At initial culture preparation, both microorganisms were cultured in Nutrient Broth Agar medium. For starter preparation, both microorganisms were maintained on a medium having composition: glucose: 130 gL^{-1}; KH_2PO_4: 1 gL^{-1}; $MgSO_4$: 7 gL^{-1}; H2O: 0.5 gL^{-1} and the cells were grown at a temperature of ± 35 °C.

2.3 Production medium and fermentation

The fermentation medium was from palmyra sap, which was collected from Gresik, East Java, Indonesia. The fresh palmyra sap was filtered and autoclaved at the temperature of 121 °C and the pressure of 15 psi. Fermentation medium was conditioned in several pH based on variables by adding NaOH 1N solution. The medium was inoculated with several different inoculum concentrations based on experimental design. Fermentation was carried out in batch condition using bioreactor with 1.8 L total volume and 1 L working volume, and well agitated. The fermentation runs for 72 - 90 h depends on the glucose concentration left in the broth.

2.4 Analytical method

The number of cells was estimated by using haemacytometer. Ethanol concentration was analysed by Gas Chromatography Scientifix GC ULTRA, detector DSQ II, and column MS 220.

2.5 Experimental design and optimization

In order to determine the optimum level of pH and inoculum concentration for ethanol production, central composite experimental design was used in the optimisation of ethanol production for both S. cereviciae and Z. mobilis. pH (X_1) and inoculum concentration (cell/mL per sugar concentration, X_2) were chosen as independent variable, ethanol concentration (gL^{-1}, Y_1) was chosen as output variable.

3. Result and discussion

3.1 Optimisation of fermentation by Saccharomyces cerevisiae

The pH and inoculum content significantly affected the batch fermentation with palmyra sap as the substrate (Dasgupta et al., 2013). Using CCD, 13 experiments were conducted with different pH and inoculum content as showed in table 1.

Table 1: Independent variables for the experiment design

No.	Variable	Coded Level				
		-1.4	-1	0	1	1.4
1.	pH	3.59	4	5	6	6.41
2.	Ratio of cell number and substrate sugar content (Saccharomyces cereviciae) (× 10^6 cell/g glucose)	2.405	2.627	3.164	3.700	3.922

Ethanol content response was observed after the reducing sugar was completely consumed or after maximum fermentation time of 90 h. Response was analysed using ANOVA method and model estimation analysis was

used using Lack of Fit Test. There are three prediction models that could be predicted as linear, two factor interaction and full quadratic. From the Lack of Fit analysis, P value > 0.05 was obtained, thus full quadratic model was significant. Second order polynomial for ethanol content prediction is shown in Eq(1).

$$Y = -270.675 + 20.918\ X_1 + 1.086\ X_2 - 1.835\ X_1^2 - 0.001\ X_2^2 - 0.004 X_1 X_2 \quad (1)$$

Table 2: Statistical significance of regression coefficient for ethanol production from Palmyra sap by Saccharomyces cerevisiae

Factor	Coefficient	F-value	P-value
Intercept	-270.675		0.003
pH	20.917	2.45	0.162
Inocculum content	1.621	37.14	0.000
pH x pH	-1.835	3.04	0.125
Inocculum content x Inocculum content	-0.002	40.65	0.000
pH x Inocculum content	-0.006	0.05	0.822

Statistical model significance was tested using ANOVA method and presented in Table 2. From the table above, there are two linear factors, those are two quadratic factors and one interaction factor. It shows that the inoculum content and the quadratic factors are significant. pH, quadratic factor of pH and interaction factor of pH is not significant. Natural pH range of palmyra sap is 4 - 6 and it gives insignificant effect to the fermentation rate, or synthesis and aromatic substances release. Constraints in fermentation will occur in very low pH (< 3.0) (Hajar et al., 2012).

Regression analysis gave value of R^2 = 0.9201 which means 92.01 % of the sample variation in ethanol content is related to independent variable. It can be said that regression model is suitable to predict optimum ethanol content because there is a small difference between experimental and predictive value. (Chrisnasari et al., 2011). Based on the regression coefficient, optimum pH value, inoculum content, and ethanol content were predicted. Optimum value of pH 5.05 and inoculum content of 3,976,760 cell.mL^{-1}/glucose content were obtained. Optimum ethanol content was obtained in 104,452 g/L value. Optimisation with RSM was presented in Figure 1.

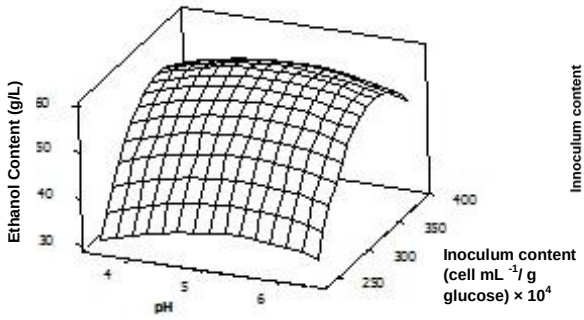

Figure 1: Response surface graph for ethanol content optimisation for Saccharomyces cerevisiae

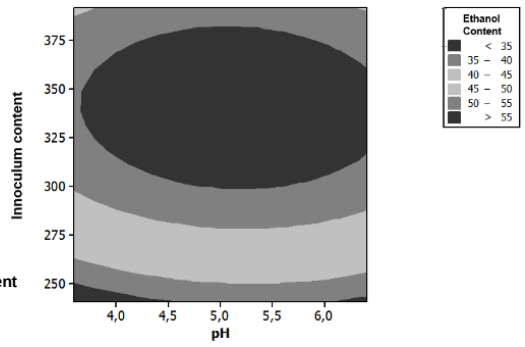

Figure 2: Contour plot for ethanol content optimisation for Saccharomyces cerevisiae

Optimum pH of 5.05 suits the value shown in various references about optimum pH for fermentation by Saccharomyces cerevisiae. Ghosh et al. (2011) conducted a research for optimum pH and the value of 5.5 was obtained. In this research, yeast cell number per palmyra sap glucose content was chosen as the variable. From the optimisation result, 3,976,760 cell.mL^{-1}/g glucose is the optimum inoculum content to produce the highest ethanol content. It is shown in Figure 1 that the ethanol concentration will increase at first, but it will inverse right after reaching its optimum content. According to Chrisnasari et al. (2011), ethanol content will increase as the inoculum content added to fermentation process increases, but it will be inversed after it reaches its optimum content due to nutrient deficiency.

The graph shows plateau line in pH variable which indicates that level of variation in pH variable does not really affect the observed system. Curvature exists in inoculum content variable shows that level of variation in this variable matters. The highest surface point for inoculum content and pH are situated in the experimental area range, meanwhile the highest surface point for ethanol content is located outside the experimental area

range which means that ethanol production can be optimised outside the experimental area range (Bezerra et al., 2008).
Based on the optimisation method, the optimum value of ethanol content is 104.452 g/L. This value confirms the pattern shown in contour plot where ethanol yield can be optimised above 55 g/L (Figure 2). This optimisation is constrained by the maximum ethanol yield of 0.51 and the maximum durability of yeast in respond to ethanol content which is 12 %v/v (Dombek and Ingram, 1987). For those reasons, a limit was set according to the constrained optimisation. A maximum value of inoculum content was obtained at the value of 3,973,760 cell.mL^{-1} / gL^{-1} glucose, yielding a predictive ethanol concentration of 94.68 %.

3.2 Optimisation of fermentation by Zymomonas mobilis

The pH and inoculum content are the most influential factors in batch fermentation with palmyra sap as the substrate (Dasgupta et al., 2013). Central Composite Design (CCD) is used to find the optimum value with different pH and inoculum content combination as shown in table 3.

Table 3: Independent variables for the experiment design for Zymomonas mobilis

No.	Variable	Coded Level				
		-1.4	-1	0	1	1.4
1.	pH	3.59	4	5	6	6.41
2.	Ratio of cell number and substrate sugar content (Zymomonas mobilis) (× 10^6 cell/g glucose)	1.172	1.394	1.930	2.466	2.689

Ethanol content response was observed after reducing sugar content has depleted or 90 h maximum fermentation time. Response was analysed using ANOVA method and model estimation was conducted using Lack of Fit test. There are three possibilities of model: linear, two factor interaction, and full quadratic. From Lack of Fit analysis, P value > 0.05 was obtained, thus quadratic model was significant. Second order polynomial equation to predict ethanol content is:

$$Y = -69.2320 + 32.4744\ X_1 + 0.1779\ X_2 - 2.9785\ X_1^2 + 0.0004\ X_2^2 - 0.0037 X_1 X_2 \quad (2)$$

Table 4: Statistical significance of regression coefficient for ethanol production from Palmyra Sap by Zymomonas mobilis

Factor	Coefficient	F-value	P-value
Intercept	-69.2320		0.003
pH	32.4744	2.27	0.176
Inocculum content	0.1779	0.25	0.632
pH × pH	-2.9785	2.35	0.169
Inocculum content x Inocculum content	0.0004	0.36	0.568
pH × Inocculum content	-0.0037	0.01	0.941

Statistical model significance was tested using ANOVA method and presented in Table 4. There are two linear factors, two quadratic factors, and an interaction factor. All factors are proven not significant because of P value > 0.05. Research from Sivasakthivelan et al. (2014) concluded that ethanol yield was significantly increased in pH 4 to 6.5. Optimum ethanol production can be achieved in this pH range. Optimum fermentation pH by Zymomonas mobilis is 6.5 which is outside the variable range in this research. Chrisnasari et al. (2011) obtained a result of optimum inoculum concentration of 23.05 % (v/v) and there is no other research using inoculum content variable in cells/mL per g/L glucose unit.
From the regression analysis, the value of R^2 = 0.9290 was obtained. It indicates that 92.90 % of sample variation was related with the independent variable, hence the model is suitable. Based on the regression coefficient, pH, inoculum content, and ethanol content were predicted. Optimum pH value was 5.57 and inoculum content was -1,965,972 cell.mL^{-1}/glucose. The optimum ethanol content was obtained at the value of 3.78 g/L. Optimisation with RSM was presented in Figure 3(a).

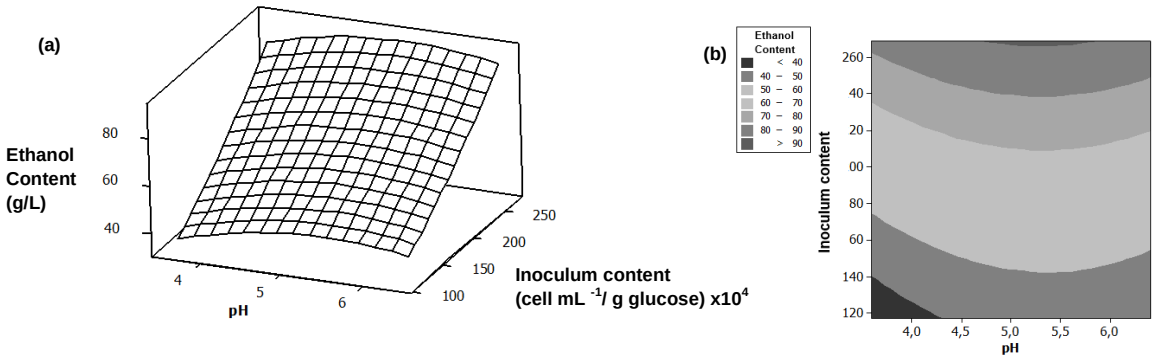

Figure 3: (a) Response surface graph for ethanol content optimisaton by Zymomonas mobilis; (b) Optimisation contour plot for ethanol content by fermentation of Zymomonas mobilis

This optimum value of pH was in agreement with previous researches on the optimum pH of fermentation by Zymomonas mobilis. Sivasakthivelan et al. (2014) wrote that ethanol yield increases in pH range of 4 to 6.5 thus ethanol production is optimum in this range. For the batch fermentation by Zymomonas mobilis, microorganism exhibit less sensitive response towards pH in the range of 5.0 - 7.5, thus pH is not significantly influential (King and Hossain, 1982). Optimisation shows -1,965,972 cell.mL^{-1}/g as the optimum inoculum content because the graph showed a saddle surface. In the saddle surface there is an existence of both maximum and minimum relative point, thus optimum value can be determined by visual inspection in the highest point of the graph (Bezerra et al., 2008). Saddle point could not represent optimum value. It is concluded that the optimum pH is 5.57 and inoculum content is 2,660,000 cells/mL according to the growth curve, yielding a predictive ethanol concentration of 94.68 %. Optimum ethanol content can be determined from Figure 3(b). Figure 3(b) shows that ethanol content can be optimised up to above 90 g/mL.

3.3 Comparison of Saccharomyces cerevisiae and Zymomonas mobilis fermentation

A validation run is carried out to confirm the result from optimisation design. The results are presented in Table 5.

Table 5: Comparison from validation run result

No.	Microorganism	pH	Innoculum Content (cell.mL^{-1}/g.mL^{-1} glucose)	Ethanol Concentration, Predictive (g/L)	Ethanol Concentration, Experimental (g/L)
1.	Saccharomyces cerevisiae	5.05	3,976,760	94.68	85.60
2.	Zymomonas mobilis	5.57	2,660,000	94.68	94.75

For pH value, the optimum value is at 5.05 for Saccharomyces cerevisiae and 5.57 for Zymomonas mobilis. This shows that both microorganisms do not have clear distinction in terms of their optimum pH. This is due to the fact that at a pH range of 5.0 to 7.5, Zymomonas mobilis has excellent durability and the variation of pH does not affect its performance significantly (King and Hossain, 1982). Saccharomyces cerevisiae optimum pH for ethanol production is 5.0 to 5.2, which is consistent with our result (Narendranath and Power, 2003). This results shows that Zymomonas mobilis has a better overall performance despite a large variation of pH, both in acidic and alkaline condition.

The second parameter is the inoculum content. Saccharomyces cerevisiae needs a denser population of cells to produce lower concentration of ethanol in comparison to Zymomonas mobilis. This is due to the higher resistance of Zymomonas mobilis to high ethanol concentration because of its hopanoid structure or complex lipid membrane which makes its cell walls denser and more stable, causing ethanol molecules to be constrained from entering its cell wall (Widjaja et al., 2015). Saccharomyces cerevisiae produce lesser concentration of ethanol due to its inability to entangle xylose to ethanol despite its excellence in decomposing glucose to ethanol (Widjaja et al, 2016).

4. Conclusion

There are differences in the optimum pH level, which is 5.05 for Saccharomyces cerevisiae and 5.57 for Zymomonas mobilis. Distinct difference was found in the optimum inoculum content which was 3,976,760

cells.mL^{-1}/gL^{-1} glucose for Saccharomyces cerevisiae and 2,800,000 cells.mL^{-1}/gL^{-1} glucose for Zymomonas mobilis. Optimum fermentation condition for fermentation using Saccharomyces cerevisiae produces 85.60 g/L of ethanol where fermentation using Zymomonas mobilis produces 94.75 g/L of ethanol. It can be concluded that despite the lacking of significance in its optimization model, Zymomonas mobilis still carries a better overall fermentation performance.

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