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OPTIMIZATION OF PALMYRA PALMSAP FERMENTATION USING CO-CULTURE OF *Saccharomyces cerevisiae* and *Pichia stipitis*

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ABSTRACT

Palmyra palm sap whose main sugar components can be used for the food grade ethanol feedstock is potential to increase economic value. Therefore, its production process needs to be improved especially in fermentation process. This study aims to investigate the best condition of the fermentation of palmyra palm sap to be ethanol using co-culture of *Saccharomyces cerevisiae* and *Pichia stipitisin* different variables such as pH, initial inoculum and sugar concentration to get the best ethanol fermentation yield. The experiment was designed using statistical method which is Response Surface Methodology (RSM) and carried out in batch-wise with a working volume of 100 mL for 80 hours. The coefficient of pH and inoculum as a linear form and all quadratic coefficients have remarkable effect on the ethanol yield (P value < 0.05). The fit of model gave high value of R^2 of 0.983, indicated that 98.3% of the variability in the response could be explained by the model. The highest ethanol yield was obtained 0.32 (g ethanol/g total sugar) with efficiency = 65.42% at pH 5.28, inoculum concentration of 6658612(cell.ml^{-1})/(g.l^{-1}), and sugar concentration of 120 g/l.

Keywords: Co-culture, ethanol, fermentation, palmyra palm sap, response surface methodology.

INTRODUCTION

Food grade ethanol is well-known as one of the most versatile products which can be used as one of the materials for food and beverage industry, pharmaceutical products, cosmetics and personal care, as well as for medical and laboratory purposes. Its production is relatively smaller than others, but it has the highest selling value [1]. The food grade usually has 96% of ethanol content and should be free of ketones, fatty acid, esters, aldehydes, and so on. Those impurities caused unpleasant odour and flavour, due to the toxic which caused health problems [1]. Food grade ethanol can be produced from fermentative processes of microbial metabolism, which was for the transformation of several raw materials in product, such as sugar based [2], starch based [3], and lignocelluloses based materials [4].

Palmyra palm tree (Borassus flabellifer) is a native to South and Southeast Asia such as Cambodia, India, Indonesia, Malaysia, Myanmar, and Thailand [5]. Palmyra palm sap from palmyra palm tree is one of potential sugar based raw materials for ethanol feedstock substitute which is abundantly available in Indonesia especially in coastal area of East Java. In Tuban-East Java. The planting area of Palmyra palm trees reaches1, 183 hectares in 2013 and it can be taped through out of the year. The fresh sap is a low price drinking juice known as "legen" with relatively high sugar content from 13 to 18 g/100 ml[5] and complete nutrition for the growth of microorganisms such as sugar, protein, nitrogen, mineral, and vitamin B complex.

Previous studies have investigated the ethanol production by fermentation process using yeasts, bacteria and sugar based material source as well as the derivative products of palmyra palm tree. *Saccharomyces cerevisiae*, *Pichia stipites*, and *Zimomonas mobilis* have been reported to be able to produce ethanol by Palmyra palm sap fermentation [6-8]. S. cerevisiae, known as sugar yeast,is from a genus of kingdom fungus. It has widely been used for fermentation because of its high ethanol tolerance and fast fermentation rates which produced a high ethanol yield [9]. Meanwhile, P. stipitis is a yeast from genus *Schefferomyces* which is included in groups of yeast that was isolated from rotting wood and insect larvae in wood [10]. Itcan produce ethanol without oxygen presence [11] and ferment glucose, xylose, mannose, galactose, and cellobiose [12], but itis preferred glucose as material for fermentation to xylose, where its consumption rate of glucose is higher than of xylose in the same condition [13]. Z. mobilis has Entner Doudoroff (ED) pathway and can achieves 5-10% higher yield of ethanol with productivity up to 2.50 times higher than traditional yeast fermentation [14], but it produces a low amount of biomass compared with *S. cerevisiae* [15].

Maximum ethanol production can be obtained by standardization and optimization of the fermentation process using experimental design method such as the Respond Surface Methodology (RSM) and Central Composite Design (CCD). In food and science technology, RSM has been widely used for optimization due to its comprehensive theory, high effectiveness, and its simplicity [16]. It can determine the desired operating areas on the factors that affect the response [17]. Whilst, CCD can provide the same prediction to all points from the center [18]. Optimization on fermentation of palmyra palm sap has been conducted by Z. mobilis in three independent variables namely sugar concentration, urea concentration, and inoculum content by [19].[8]has used *S. cerevisiae* to ferment palmyra palm juice in difference of pH, temperature, and incubation time as experimental independent variables. Two independent variables, pH and inoculum concentration, were chosen in Palmyra palm sap fermentation by *S. cerevisiae* and *Z. mobilis* [6].

In this research, the influence of three independent variables (pH, inoculum and initial sugar concentration) and their interaction was studied to find the optimal condition of ethanol production from palmyra

palm sap by co-culture of *S. cerevisiae* and *P. stipitis*. The experiment was designed using RSM with CCD.

MATERIAL AND METHOD

Preparation of palmyra palm sap

The fresh palmyra palm sap for fermentation medium was collected from Tuban, East Java, Indonesia. It was filtered and sterilized at 121°C and 15 psi for 15 minutes. Its sugar content was measured using Dinitrosalicylic Acid (DNS) reagent as described by[20]. The fresh Palmyra palm sap was stored at 4°C for stock.

Inoculum preparation

S. cerevisiae and P. stipitis were gained from Industrial Microbiology Laboratory, Department of Chemical Engineering, ITS Surabaya, Indonesia. They were then inoculated from slants on to fresh potato dextrose agar medium (40 g/l) and incubated at 30°C for 3 days. Furthermore, one loop of S. cerevisiae and P. stipitis was inoculated into the preculture medium containing: 180 ml of sterile Palmyra palm sap; 1 (g/l) of KH_2PO_4; 0.5 (g/l) of $MgSO_4.7H_2O$; 1 (g/l) of $(NH_4)_2SO_4$; and 10 (g/l) of yeast extract. The cultures were incubated at 30°C and 120 rpm and drowned every single hour for the measurement of cell growth using Hemacytometer[21]. By plotting the number of cell against a time, the log phase for culture growth could be determined. The cell number as the variables were designed in the range of log phase, thus in time of the cell number was achieved, 1 of ml culture was inoculated to 100 ml of sterile Palmyra palm sap. The culture growth was monitored during the fermentation process by counting the number of cell in the sample every 8 hours.

Experimental design

Fermentation medium was made from the dilution of palmyra palm sap using lesssugar condition (110, 120, and 130 g/l). Furthermore, adjustment of the pH of the medium to several variables was conducted by adding NaOH or HCl 1 N solution. The medium was inoculated with several different inoculum concentrations based on experimental design. Fermentation was carried out in batch condition using some bottles with 160 ml of total volume and 100 ml of working volume. The bottles were incubated in incubator shaker at 32°C and 100 rpm for 80 h.

Three factors, involving pH (X_1), inoculum concentration (X_2, $(cell.ml^{-1})/(g.l^{-1})$, and Sugar concentration (X_2, g/l), were selected to be independent variables and yield (Y) was used as the response. A 2^3 factorial Central Composite Design, with six axial points ($\alpha=\sqrt{3}$), and five replications at the center points which lead to a total number of 19 experiments. The coded level of each factor in the CCD can be seen in Table-1.

The model corresponded to the central data on the response of ethanol yield was expressed in second-order polynomial function, shown in Eq. (1):

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

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

Analytical methods

To calculate the yield fermentation processes, determination of the amount of initial and residual sugar concentration was needed. It was estimated using DNS method in which the standard curve of sugar (glucose) was obtained by spectrophotometer at λ = 540 nm. The Observed yield and efficiency were obtained by Eq. (2) and (3) respectively.

$$\text{Observed Yield (Y)} = \frac{Weight\ of\ etanol\ from\ fermentation\ process}{Weight\ of\ initial\ sugar} \quad (2)$$

$$\text{Efficiency} = \frac{Fermentation\ Yield}{Theoretical\ Yield} \times 100\% \quad (3)$$

The efficiency shows how much the ethanol was produced from useable sugar in the fermenter. While, the theoretical yield of ethanol was determined 0.51 [22]. That value was obtained from the fermentation reaction mechanism of sugar (Eq. (4)) to be ethanol which is shown in Eq. (5):

$$C_6H_{12}O_6 + \text{cell mass} \rightarrow 2\ C_2H_5OH + 2\ CO_2 + \text{more cell mass} \quad (4)$$

$$\text{Theoretical Yield} = \frac{Weight\ of\ ethanol\ (product)}{Weight\ of\ sugar\ converted\ to\ ethanol\ (reactant)} \quad (5)$$

The ethanol concentration was analyzed using Gas Chromatography Scientific GC ULTRA with detector DSQ II and column MS 220.

RESULTS AND DISCUSSIONS

A sugar content in palmyra palm sap and the number of cell

Thesugar content in palmyra palm sap was identified of 139.42 g/l. To achieve the sugar value as the experimental variable, Palmyra palm sap was diluted by sterile distilled water, then analyzed using DNS reagent and determined by plotting to standard sugar (glucose) curve whose its statistical parameters of liner regression is shown in Table-2.

Table-1. Coding and the symbol of independent variables in different level of the CCD ($\alpha = \sqrt{3}$).

Factor (unit)	**Symbol**	**Coded level (Z_i)**;i= subscript for factor's code				
		-α	**-1**	**0**	**+1**	**+α**
pH	X_1	3.77	4.5	5.5	6.5	7.23
Inoculum concentration ($cell.ml^{-1}$)/($g.l^{-1}$ of sugar)	X_2	6171875	6400000	6712500	7025000	7253125
Sugar concentration (g/l)	X_3	102.68	110	120	130	137.32

Table-2. Statistical parameters of the standard curve for glucose (liner regression).

Parameters	**Glucose** (g/l)	**Absorbance** (λ = 540nm)
Variable	3.7060	2.452
	2.948	2.159
	2.2236	1.6624
	1.4824	1.0944
	0.7412	0.7022
	0	0
Slope		1.4098
Intercept		0
Correlation coefficient (R^2)		0.985

The cell concentration was obtained by using counting chamber called hemocytometer. Determination of cell number in medium is probably difficult to be totally same as the recommended value from statistical software. Thus, it was applied responsible approximation of cell number value, paying attention to considering when this approximation was in the log phase and reached the expected cell number.

Optimization of fermentation using co-culture

Three variable factor designs, involving pH, inoculum concentration, and sugar concentration, were studied in different level to find out optimum condition on the ethanol produced. Responses were taken after the fermentation process was done, based on the remaining sugar content in the substrate.

Lack of Fit Test was used to analyze model estimation. Lack of fit was the condition where the simple linear regression did not correspond to the Figures [8]. In this case, the P value for lack of fit test were 0.61 (P> 0.05) which means that the lack of fit model is not significant and the equation used in this experiment was appropriate. If P value from lack of fit model is less than 0.05, we need more complex model for this experiment. Thus, we can conclude that, the full quadratic model which was used in the experiment design was valuably significant on the statistic. The second order polynomial equation that was used for predicting ethanol yield ($\hat{Y}$) is:

$$\hat{Y} = -32.349 + 2.8641 \times 10^{-1}. X_1 + 8.9169 \times 10^{-6}. X_2 - 8.4124 \times 10^{-2}. X_3 - 6.3319 \times 10^{-2}. X_1^2 - 6.8910 \times 10^{-13}. X_2^2 - 5.5383 \times 10^{-4}. X_3^2 + 5.0240 \times 10^{-8}. X_1X_2 + 3.9500 \times 10^{-4}. X_1X_3 + 6.9360 \times 10^{-9}. X_2X_3 \quad (6)$$

In Table-3, the predicted of ethanol yield gave a small error (0.001297) to determine the observed ethanol yield. The highest efficiency was obtained 65.42%. Table-4 shows the analysis of variance for experimental response. The significant influence of the factor on statistical second order model equation (Eq. 6) was clarified by an F-test. Table-4 reveals that the X_1, X_2 as a linear form and quadratic coefficient of X_1^2, X_2^2, X_3^2, X_1*X_2,andX_2*X_3 have remarkable effect on the ethanol yield. From F value, those have given significant effect due to their interaction of each component (P value < 0.05). Meanwhile, X_3 and X_1*X_3 did not give significant effect because the P value was bigger than 0.05. But, the overall F value was significant, thus the model was considered as significant. The X_3 and X_1*X_3 interaction set as variable did not give significant effect at the 5% probability level [18].

In the Figure-1, the red line shows the linear regression for the predicted yield and observed yield. That fit of model gives high R^2 value of 0.983, indicates that 98.3% of the variability in the response can be explained by the model.

From the response surface graph, Figure-2, we can see that in the same inoculum concentration with different pH will affect the ethanol production. This is due to the effect of pH of the substrate on microorganism enzyme activities [8]. Also, if the amount of inoculum

concentration decreased, the amount of ethanol yield would decrease, and vice versa. However, there was a point where the maximum inoculum was needed. The addition of inoculum would decrease the produced ethanol because of limited nutrition for growth of the microbes [19]. The optimum ethanol yield was obtained 0.32 (g ethanol/g total sugar) at pH 5.28, with inoculum concentration of 6658612 (cell.ml^{-1})/(g.l^{-1}).

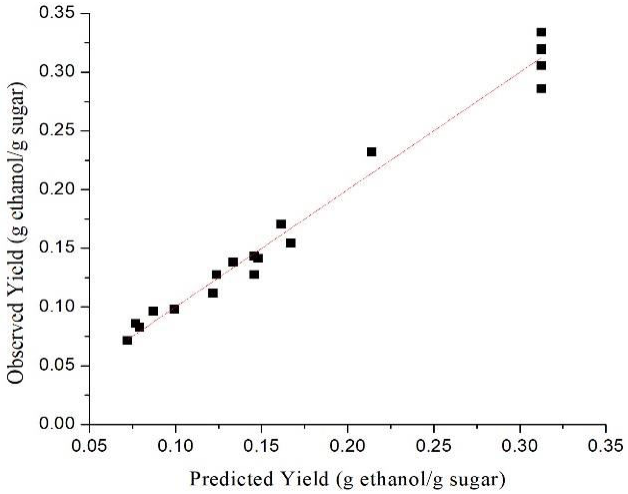

Figure-1. Charts of predicted values versus observed values of ethanol produced from palmyra palm sap.

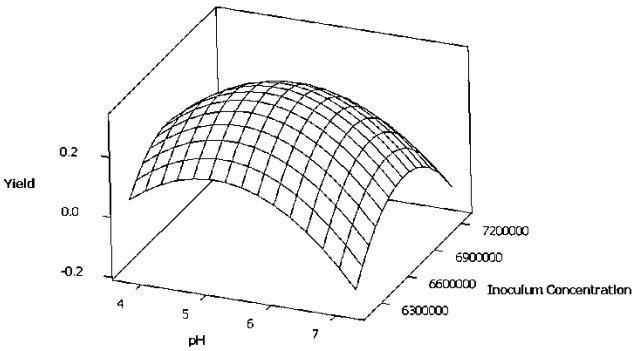

Figure-2. Response surface graph of yield affected by pH and sugar concentration.

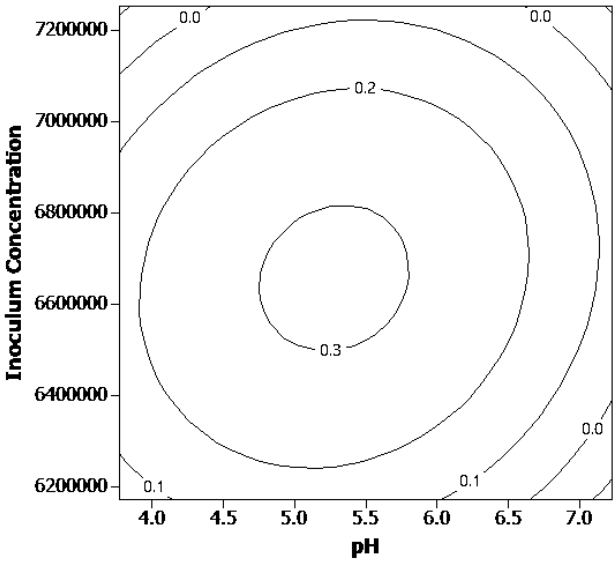

Figure-3. Contour plot showing yield in response to varying pH and inoculum concentration.

Figures 4 and 5 show the interaction between inoculum concentration and sugar concentration. From the response surface graph, we can see that if the amount of inoculum concentration is decreased the amount of ethanol yield will decrease too, and vice versa. From Figure-5, when **t**he optimum ethanol yield is obtained 0.32 (g ethanol/g total sugar), the optimum sugar concentration is 120 g/l.

Table-3. The Design and results of the CCD experiment.

Run	Z_1	Z_2	Z_3	Observed yield (Y)	Predicted yield ($\hat{Y}$)	Ethanol concentration (% v/v)	Ethanol concentration (g/L)	Efficiency (%)
1	-1	-1	-1	0.2320	0.2142	3.2345	25.5202	45.4902
2	1	-1	-1	0.1271	0.1240	1.7722	13.9827	24.9216
3	-1	1	-1	0.0980	0.0996	1.3633	10.7564	19.2157
4	1	1	-1	0.0710	0.0721	0.9899	7.8103	13.9216
5	-1	-1	1	0.1701	0.1616	2.8023	22.1101	33.3529
6	1	-1	1	0.0961	0.0872	1.5838	12.4962	18.8431
7	-1	1	1	0.1379	0.1337	2.2725	17.9300	27.0392
8	1	1	1	0.1116	0.1221	1.8389	14.5089	21.8824
9	-α	0	0	0.1542	0.1673	2.3450	18.5021	30.2353
10	α	0	0	0.0826	0.0793	1.2562	9.9114	16.1961
11	0	-α	0	0.1273	0.1459	1.9365	15.2790	24.9608
12	0	A	0	0.0857	0.0769	1.3036	10.2854	16.8039
13	0	0	-α	0.1412	0.1482	1.8375	14.4979	27.6863
14	0	0	A	0.1431	0.1459	2.4900	19.6461	28.0588
15	0	0	0	0.3055	0.3128	4.6468	36.6633	59.9020
16	0	0	0	0.3199	0.3128	4.8657	38.3904	62.7255
17	0	0	0	0.3337	0.3128	5.0748	40.0402	65.4314
18	0	0	0	0.2860	0.3128	4.3498	34.3199	56.0784
19	0	0	0	0.3190	0.3128	4.8517	38.2799	62.5490

Table-4. The Analysis of Variance for the Result of The CCD experiment.

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	0.148998	0.148998	0.016555	57.97 s	0
Linear	3	0.014628	0.014628	0.004876	17.07 s	0
X_1	1	0.009065	0.009065	0.009065	31.75 s	0
X_2	1	0.005556	0.005556	0.005556	19.46 s	0.002
X_3	1	0.000006	0.000006	0.000006	0.02 ns	0.889
Square	3	0.128515	0.128515	0.042838	150.01 s	0
$X_1 * X_1$	1	0.032047	0.032047	0.059456	208.21 s	0
$X_2 * X_2$	1	0.050981	0.050981	0.067157	235.17 s	0
$X_3 * X_3$	1	0.045487	0.045487	0.045487	159.29 s	0
Interaction	3	0.005855	0.005855	0.001952	6.83 s	0.011
$X_1 * X_2$	1	0.001972	0.001972	0.001972	6.91 s	0.027
$X_1 * X_3$	1	0.000125	0.000125	0.000125	0.44 ns	0.525
$X_2 * X_3$	1	0.003758	0.003758	0.003758	13.16 s	0.006
Residual Error	9	0.002570	0.002570	0.000286		
Lack-of-fit	5	0.001273	0.001273	0.000255	0.79 ns	0.61
Pure Error	4	0.001297	0.001297	0.000324		
Total	18	0.151568				

R^2 = 0.983;s (significant (P < 0.05 and P < 0.005)); ns (not significant (P>0.05))

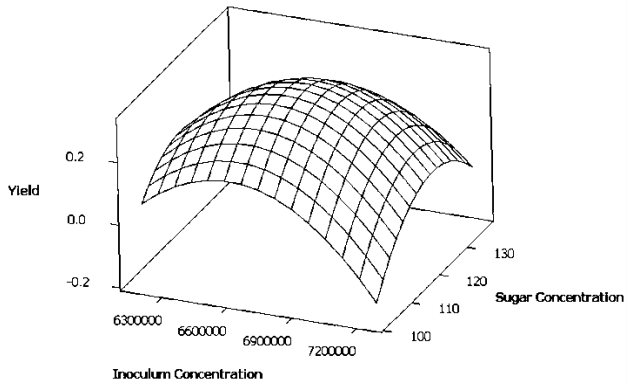

Figure-4. Response surface graph of yield affected by inoculum and sugar concentration.

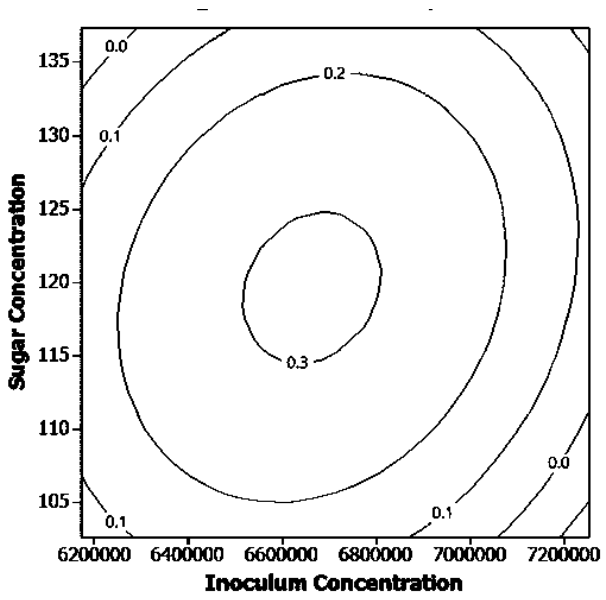

Figure-5. Contour plot showing yield in response to varying inoculum and sugar concentration.

For *Saccharomyces cerevisiae*, ethanol was produced when the sugar concentration was relatively low, even at the anaerobic condition [23]. Pichia stipitis is a microbe that can tolerate high sugar concentration, but cannot tolerate ethanol at high concentration [24]. Thus, the co-culture between *Saccharomyces cerevisiae* and *Pichia stipitis* could ferment higher sugar concentration.

Figures 3, 6, and 7 show the interaction between sugar concentration and pH. To get optimum yield, the optimum number of inoculum and sugar concentration should be fermented in pH 5.28. According to [23], an optimum ethanol yield could be obtained if the range of pH for the fermentation using *P. stipitis* was around 4.5-5.5. Meanwhile, [25] explained, an optimum ethanol yield could be achieved if the pH range for the fermentation by *S. cerevisiae* was around 5.0-5.5. Thus, it was suited for both microorganisms. The sugar concentration required for the optimum ethanol yield was 120 g/l. For *S. cerevisiae*, ethanol was produced when the sugar concentration was relatively low, even at the anaerobic condition [23]. *P. stipitis* is a microbe that can tolerate high sugar concentration, but cannot tolerate ethanol at high concentration [24]. Hence, the mixed culture between *S. cerevisiae* and *P. stipitis* could ferment higher sugar concentration.

From the experiment, the optimum condition for fermenting palmyra palm sap using co-culture with the highest yield of ethanol was obtained at 0.32 (g ethanol/g total sugar) with pH 5.28, sugar concentration of 120 g/l, and inoculum concentration of 6658612 (cell/ml^{-1})/(g.l^{-1}).

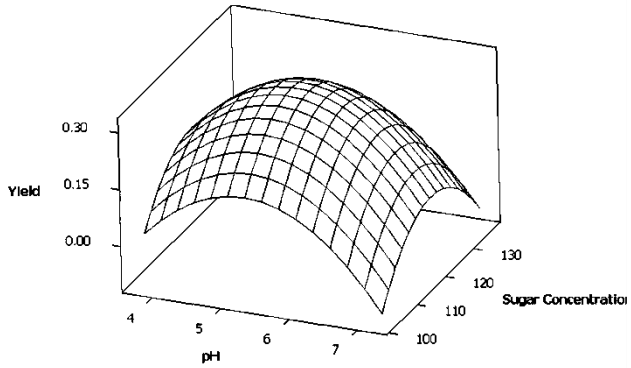

Figure-6. Response surface graph of yield affected by pH and sugar concentration.

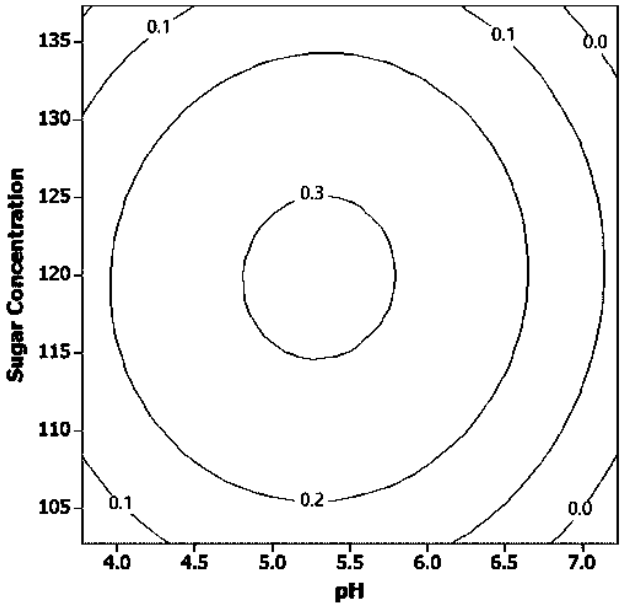

Figure-7. Contour plot showing yield in response to varying pH and sugar concentration.

This is in line with the earlier research by [26]where the use of co-culture of *P. stipitis* and *S. cerevisiae* gave better results than the monoculture of *Saccharomyces cerevisiae*, the ethanol concentration was higher than resulted in this present study. Due to different use of substrate such as Sweet Sorghum Sap, which has xylose content that can be fermented by *P. stipitis*. Forsugar based ingredient use like palmyra palm sap;

these two microbes produced a good ethanol concentration.

In the same time, we also conducted an experiment on ethanol production from palmyra palm sap fermented by *Saccharomyces cerevisiae* as the control. The result shows that the highest ethanol yield was obtained 0.2368 (g ethanol/g total sugar) at pH 4.8, sugar concentration of 110 (g/l), and inoculum concentration of 12.740.970 (cell.ml^{-1}) / (g.l^{-1}). Its yield was lower than using co-culture between *S. cerevisiae* and *P. stiptis* whose a good fermentation performance.

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 palm sap with high fit of model value (R^2 = 0.983). The optimum fermentation condition was of 5.28, 6658612 (cell.ml^{-1}) /(g.l^{-1}), and 120 g/l. for pH, inoculum, and sugar concentration respectively. The optimum ethanol yield was of 0.32 (g ethanol/g total sugar) with efficiency = 65.42%. This experimental result was significantly higher than the previous studies of palmyra palm sap fermentation.

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