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Original Article

Alternative energy under the Royal Initiative of His Majesty the King: Ethanol from nipa Sap using isolated yeast

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Abstract

The objective of this work was to add value to nipa sap from nipa palm. Nipa palm is a plant grown in a conservation program under a Royal Initiative Project. The potential of the nipa sap was assessed for bioethanol production. The sap was fermented using the yeast isolated from the fresh sap. The fermentation variables including total sugar concentration, pH, yeast suspension, temperature, and time were analyzed with response surface methodology on the ethanol concentration, ethanol yield, and fermentation efficiency. Time was the most significant effect, whereas the yeast suspension had the least effect. The yield is considered the priority. The optimum total sugar concentration was 212.6 g/l with a pH of 4.8, and 10^6 cells/ml of yeast suspension at 32 °C for 77 h. An experimental yield of 53.2% was achievable under these conditions. The achieved experimental concentration of ethanol was 113.1 g/l. Ethanol productivity and efficiency were 1.5 g/l h and 98.6%, respectively.

Keywords: ethanol, nipa sap, yeast isolated, fermentation, Royal Initiative Project

1. Introduction

Energy sources are one of the key factors driving the economies worldwide. However, the consumption of petroleum-based fuels, which are the major sources of energy around the globe, has also caused a great amount of environmental pollution. One of the suitable approaches to reduce the current problem is alternative sources of energy (Lang *et al.*, 2001). Ethanol is an alternative source of energy and has become a prominent biofuel since its properties are similar to gasoline (Costa & Sodre, 2010) and its use offers the promise of an improvement in the agricultural economy (Gnansounou & Dauriat, 2010; Wang, 2011). Ethanol can be produced from biomass and agricultural residues such as sweet sorghum (Fernandes *et al.*, 2014), corncob (Zhang *et al.*, 2010), bagasse (Costa *et al.*, 2015), palm trunk (Bansal *et al.*, 2016), and oil palm frond juice (Srimachai *et al.*, 2015). At present, the largest producers and consumers of ethanol from corn starch and sugarcane are the U. S. and Brazil, respectively. In Thailand, feedstocks for the production of ethanol are cassava and sugarcane which still have some limitations in their need for water, fertilizers, pesticides, machinery, and electrical power. Meanwhile, waste products, especially from sugarcane such as leaves and bagasse, are a concern after extraction of the juice (Luo *et al.*, 2009). At the moment, compared with that of gasoline, the cost of ethanol production is not economically competitive due to its relatively high cost of feedstocks, i. e. cassava chips, molasses, and sugarcane juice, which can also be used in several other industries. Thus, it is necessary to increase alternative feedstocks and develop cost-effective processes for cost reduction in the production of ethanol.

Among the potential biomass materials for ethanol production is the nipa palm (*Nypa fructicans*). It is grown throughout Asia (Jabatan, 2009) and it is of interest because it gives more sugar yield than sugarcane (Tumanaidu *et al.*, 2013). The plant grows naturally in all areas including saline soil and swamps as well as slow-moving tidal and short-term drying areas. Moreover, it is a plant already conserved and promoted in the projects initiated by Her Royal Highness

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Princess Maha Chakri Sirindhorn. This palm tree is useful for coastal rehabilitation and restoration of land after shrimp farms are abandoned. Nipa sap is collected easily by tapping the stalk and cutting off the edible inflorescence of the palm. Therefore, harvesting the sap causes no waste, no damage to the environment, and no effect on the growth of the palm (Dennett, 1972; Hamilton & Murphy, 1998). Furthermore, the palm can provide sugar-rich sap regularly without shortening the life of the tree (Gibbs, 1911).

Nipa sap also serves as raw material for the production of dried sugar, alcoholic beverages, and vinegar. The sap is rich in various kinds of sugar, organic nutrients, minerals, and microbes. Therefore, it is also an ideal raw material for bioethanol production. Although it has gained interest in fuel alcohol production, there is no intensive research on this source. Over the past few years it has been used only locally due mainly to the problems of storage and the short shelf-life. The sap becomes rancid easily by microbial decomposition (Dodic′ *et al.*, 2009; Lipnizki *et al.*, 2006; Tamunaidu *et al.*, 2013) which is the main drawback for the commercialization of ethanol made from nipa sap.

The fermentation of the sap can be carried out directly without an intermediate hydrolysis step (Abdullah *et al.*, 2015; Germec *et al.*, 2015; Gumienna *et al.*, 2016; Luo *et al.*, 2014). However, in order to obtain profitability of ethanol production, the decomposition problem needs a solution and the operating parameters of fermentation need to be investigated (Liu & Shen, 2008). The ethanol concentration, ethanol yield, and fermentation efficiency, which are affected by the operating parameters, can be determined by response surface methodology (RSM). RSM has been widely used to analyze the effects of individual process variables on the response variables. It can evaluate the interactions between different mathematical approaches widely applied in many processes (Baghkheirati & Bagherieh-Najjar, 2016; Gupta & Nayak, 2016; Gupta & Parkhey, 2014; Santos *et al.*, 2016).

The aim of this work was to add value to nipa sap from nipa palm while assessing the full potential of the nipa sap. The work may also help create an efficient alternative feedstock that can maintain good ecology together with developing a cost-effective process for sap-based bioethanol production. The sap was fermented using the yeast isolated from the fresh sap as the culture medium. The variables in the fermentation process, that included the initial total sugar concentration (106.3–318.9 g/l), initial pH (4.5–6.5), yeast suspension (10^6–10^8 cells/ml), temperature (28–40 °C), and time (10–144 h), were analyzed with RSM on the ethanol concentration, ethanol yield, and fermentation efficiency.

2. Materials and Methods

2.1 Nipa sap

Nipa sap was obtained from a plantation located in Pak Phanang, Nakhon Si Thammarat Province, Thailand. The nipa sap was harvested daily between February and October. The fresh sap was collected early in the morning and stored immediately at 4 °C until use. The chemical compositions of the fresh sap used in this study were analyzed using UV-Vis spectrophotometer which showed 25.9 g/l of reducing sugar (monosaccharide) and 343.8 g/l of non-reducing sugar (disaccharide).

2.2 Preparation

Based on our previous work (Puangpee & Chongkhong, 2016), the preparation for the sap began with filtering solid impurities from the fresh sap and adjusting the filtered sap to a pH of 4.9 with sulfuric acid solution. Then the adjusted sap was heated in an oil bath at 54 °C for 25 min.

2.3 Fermentation without nutrient supplementation

The yeast strain PSU-NS1 (Punlumpak, 2016) was isolated from the fresh nipa sap and used as the fermenting medium by the Department of Microbiology, Faculty of Science, Prince of Songkla University. This study is the first to focus on isolation of yeast from nipa sap. The isolated strain was prepared by mixing 1 ml of sap with 9 ml of 0.85% normal saline solution. The mixed solution (0.1 ml) was spread on a yeast mold (YM) agar plate and its pH was adjusted to 4.5. The yeast cultured on the YM agar was kept at ambient temperature (28–30 °C) for 48 h. It was then stored at 4 °C in a YM slant. Before use, the PSU-NS1 yeast was activated in YM agar for 48 h. The activated yeast cells were diluted with the sap substrates to give 10^6–10^8 cells/ml.

Fermentation was carried out in 250 ml air-locked flasks with a working volume of 100 ml. The prepared sap was cooled to room temperature and diluted with deionized water to obtain a substrate which contained total sugars of different concentrations. The pH value of the substrate was adjusted to the assigned initial value with sulfuric acid or sodium hydroxide solution. Then the required amount of activated yeast was added to the substrate. The flasks were placed in a shaking incubator (LabTech, LSI-3016A, South Korea) at a shaking speed of 80 rpm at various temperatures. The solutions were sampled at different times during the batch fermentation process which was conducted under anaerobic conditions. The experimental conditions are shown in Table 1.

Table 1. Experimental conditions and ethanol productivity results for nipa sap fermentation.

Experimental number (Exp. No.)	Process variables					Ethanol productivity (g/l h)
	Total sugars (g/l)	pH	Yeast (cells/ml)	Temperature (°C)	Time (h)	
1	106.3	5.5	10^7	34	77	0.7
2	159.5	5.0	10^7	31	44	1.8
3	159.5	6.0	10^6	31	44	1.4
4	159.5	6.0	10^7	31	111	0.6
5	159.5	5.0	10^6	31	111	0.7
6	159.5	6.0	10^7	37	44	1.0
7	159.5	5.0	10^6	37	44	1.1
8	159.5	6.0	10^6	37	111	0.7
9	159.5	5.0	10^7	37	111	0.3
10	212.6	5.5	10^7	28	77	1.3
11	212.6	5.5	10^7	34	10	5.8
12	212.6	5.5	10^7	34	77	1.3
13	212.6	4.5	10^7	34	77	1.1
14	212.6	6.5	10^7	34	77	1.1
15	212.6	5.5	10^7	34	77	1.3
16	212.6	5.5	10^7	34	77	1.3
17	212.6	5.5	10^7	34	77	1.3
18	212.6	5.5	10^8	34	77	1.0
19	212.6	5.5	10^7	34	144	0.5
20	212.6	5.5	10^7	40	77	0.4
21	265.8	6.0	10^7	31	44	1.8
22	265.8	5.0	10^6	31	44	2.8
23	265.8	6.0	10^6	31	111	1.0
24	265.8	5.0	10^7	31	111	1.1
25	265.8	5.0	10^7	37	44	1.7
26	265.8	6.0	10^6	37	44	1.7
27	265.8	6.0	10^7	37	111	1.2
28	265.8	5.0	10^6	37	111	1.1
29	318.9	5.5	10^7	34	77	1.9

2.4 Analytical methods

Reducing sugar concentration was analyzed by the dinitrosalicylic acid (DNS) method (Miller, 1959) and total sugar concentration (sum of reducing sugar and non-reducing sugar) was estimated by the modified phenol sulfuric method (Dubois *et al.*, 1956), using a UV-Vis spectrophotometer (UV, HP8453 with Chem-Station software).

The ethanol concentration was determined by gas chromatography, using an HP-FFAP column (GC 6890, Hewlett Packard, USA) equipped with a flame ionization detector. The oven temperature was set at 85 °C while the injector and detector were kept at 150 °C and 250 °C, respectively. The flow rate of the hydrogen carrier gas was set at 44.6 ml/min. The nitrogen flow rate was set at 25 ml/min while the flow rate of air was set at 300 ml/min.

Ethanol yield (%) is the percentage of the total sugars at the beginning that are converted to ethanol, and the fermentation efficiency (%) is the percentage of sugars used in fermentation that are converted to ethanol. The amount of total sugar used is the concentration of initial total sugar minus the concentration of residual total sugar.

The ethanol yield and fermentation efficiency were calculated as follows:

$$\text{Ethanol yield}(\%) = \frac{\text{ethanol obtained in fermentation (g)}}{\text{total sugars at the beginning (g)}} \times 100\% \quad (1)$$

$$\text{Fermentation efficiency}(\%) = \frac{\text{ethanol obtained in fermentation (g)}}{\left[\begin{array}{l}\langle 0.511 \times \text{reducing sugar used in fermentation (g)}\rangle + \\ \langle 0.538 \times \text{non-reducing sugar used in fermentation (g)}\rangle\end{array}\right]} \times 100\% \quad (2)$$

where 0.511 indicates the theoretical ethanol yield from glucose and fructose (0.511 g ethanol/g reducing sugar), and 0.538 indicates the theoretical ethanol yield from sucrose (0.538 g ethanol/g non-reducing sugar). Thus, 0.511 and 0.538 represent the conversion factors from reducing sugar and non-reducing sugar to ethanol, respectively (Sasaki *et al.*, 2014).

2.5 Statistical analysis

RSM was used to investigate the effects of the initial total sugar concentration (106.3–318.9 g/l: g of total sugars in 1 liter of substrate or the mass fraction calculated at 10–30% (w/w): g of total sugars in 100 g of substrate), yeast suspension (10^6–10^8 cells/ml), fermentation temperature (28–40 °C) and fermentation time (10–144 h) on the ethanol concentration (g/l), ethanol yield (%) and fermentation efficiency (%). The central composite design for the five factors with circumscribed type provided 27 experimental conditions and another two with repeated 3 center points (Table 1).

Microsoft Excel 2013 was used to conduct a regression analysis of the experimental data that employed a quadratic polynomial model shown in Equation 3.

$$\begin{aligned}\text{Ethanol or Yield or Efficiency} = {} & b_0 + b_1F_1 + b_2F_2 + b_3F_3 + b_4F_4 + b_5F_5 \\ & + b_{11}F_1^2 + b_{22}F_2^2 + b_{33}F_3^2 + b_{44}F_4^2 + b_{55}F_5^2 + b_{12}F_1F_2 + b_{13}F_1F_3 \\ & + b_{14}F_1F_4 + b_{15}F_1F_5 + b_{23}F_2F_3 + b_{24}F_2F_4 + b_{25}F_2F_5 + b_{34}F_3F_4 \\ & + b_{35}F_3F_5 + b_{45}F_4F_5\end{aligned} \quad (3)$$

Ethanol, yield, and efficiency are the response variables and F_1, F_2, F_3, F_4, and F_5 are the factor variables. The coefficient b_0 is the intercept while b_1, b_2, b_3, b_4, and b_5 are the linear terms. In addition, b_{11}, b_{22}, b_{33}, b_{44}, and b_{55} are the quadratic terms while b_{12}, b_{13}, b_{14}, b_{15}, b_{23}, b_{24}, b_{25}, b_{34}, b_{35}, and b_{45} are the interactions of the factors.

3. Results and Discussion

3.1 Components of nipa sap

The major compositions of the raw nipa sap included different types of fermentable sugars or total sugars which were divided into two main groups: reducing sugars or monosaccharides (glucose and fructose) and non-reducing sugar or disaccharide (sucrose). The non-reducing sugar can be digested into glucose and fructose by enzymes from yeast, namely sucrase or invertase (Gumienna *et al.*, 2016).

The raw sap contained 25.9 and 369.7 g/l of initial concentrations of reducing sugar and total sugars (sum of reducing and non-reducing sugars), respectively. The sugar-rich sap, therefore, is the source of several organisms including acid bacteria, molds, and yeasts that easily cause decomposition and spoilage of the sap.

In order to get the maximum benefit from the use of the sap and reduce the auto-hydrolysis of di- or poly-saccharides and avoid further decomposition of organic acids, the raw sap had to be collected early in the morning (before 7:00 a.m.) and placed immediately in screw-capped bottles at 4 °C to avoid oxygen and sunlight that would support the microbial decomposition. Not only can this preparation reduce the energy consumption and, subsequently, the cost of traditional drying of the sap to prevent spoilage, but it can also maintain nutrients in the sap which are often destroyed by heat.

After the sap preparation, the sugar concentrations increased to total sugars of 410.6 g/l and 61.3 g/l of reducing sugar. This assured that the preparation supported the function of the native microbial enzymes, for example, amylase from bacteria hydrolyzing dissolved starch and invertase from yeast hydrolyzing sucrose (Underkofler *et al.*, 1958).

3.2 Effects of fermentation on nipa sap

The experimental conditions are reported in Table 1. Responses of the experimental and predicted ethanol concentration, ethanol yield, and fermentation efficiency were used to establish a second degree polynomial model evaluating the effects of the variables (Table 2). The coefficients of the models, the results of the statistical analyses and the analysis of variance (ANOVA) are shown in Table 3. The determination coefficients (R^2) of the three responses indicated the accuracy of the models given in Equations 4, 5, and 6. The individual effects of variables and their interactions can be considered based on a *P*-value which points to the significance of the results. A probability (*P*-value) of less than 0.05 implied that a variable effect was significant (Wang *et al.*, 2008). For Fisher's *F*-test, the mean square regression (*F*-model) showed the mean square residual (*F*) and the extremely low probability value (*P*-model > *F*signif) which indicated that the model showed a good fit to the data.

Table 2. Effects of process variables on the ethanol concentration, the ethanol yield and the fermentation efficiency during nipa sap fermentation.

Exp. No.	Ethanol concentration (g/l)		Ethanol yield (%)		Fermentation efficiency (%)	
	Exp.	Predicted	Exp.	Predicted	Exp.	Predicted
1	50.3	51.3	47.3	45.0	89.7	79.0
2	81.2	88.3	50.9	54.1	95.4	104.7
3	62.0	62.0	38.9	38.9	73.1	73.1
4	69.7	69.7	43.7	45.3	85.2	83.0
5	76.6	76.6	48.0	48.0	90.0	90.0
6	41.9	39.3	26.3	26.6	51.5	67.3
7	49.1	49.1	30.8	30.8	99.9	100.0
8	73.5	73.5	46.1	46.1	86.9	86.9
9	29.8	22.0	18.7	18.1	38.3	45.5
10	102.6	94.5	48.3	45.8	93.7	97.4
11	58.4	53.0	27.5	26.3	86.2	72.2
12	96.7	96.6	45.5	45.5	88.9	88.2
13	80.9	80.6	38.1	37.7	73.7	68.0
14	81.4	83.0	38.3	38.6	72.8	69.9
15	96.9	96.6	45.6	45.5	86.7	88.2
16	96.6	96.6	45.4	45.5	88.8	88.2
17	96.5	96.6	45.4	45.5	88.8	88.2
18	73.4	73.4	34.5	34.5	64.8	64.8
19	75.2	82.1	35.4	36.6	67.0	72.7
20	29.7	39.1	14.0	16.5	86.1	73.7
21	78.7	85.2	29.6	30.1	62.5	63.8
22	124.2	124.2	46.7	46.7	89.1	89.1
23	116.3	116.3	43.8	43.8	82.4	82.4
24	126.8	128.0	47.7	47.3	90.4	83.3
25	76.3	74.9	28.7	27.0	64.4	75.2
26	73.6	73.6	27.7	27.7	97.4	97.4
27	132.9	124.6	50.0	46.8	99.9	99.3
28	121.3	121.3	45.6	45.7	98.9	98.9
29	147.1	147.4	46.1	48.5	87.0	89.2

Note: Exp. is Experimental.

3.2.1 Response analysis of the ethanol concentration

From the *P*-values of the ethanol concentration responses (Table 3), the individual and quadratic effects of fermentation time were the crucial influences on ethanol concentration. Additionally, the interaction effects indicated that time had a significant influence on total sugars, pH, yeast suspension, and temperature while other variables were of less significance.

$$\text{Ethanol} = -171.47 - 0.281F_1 - 109.69F_2 + 86.2F_3 + 28.74F_4 - 3.038F_5 + 0.000241F_1^2 - 14.82F_2^2 - 7.601F_3^2 - 0.828F_4^2 - 0.00648F_5^2 - 0.135F_1F_2 + 0.04501F_1F_3 + 0.0209F_1F_4 + 0.00453F_1F_5 + 12.81F_2F_3 + 5.312F_2F_4 + 0.42F_2F_5 - 2.032F_3F_4 - 0.08302F_3F_5 + 0.04591F_4F_5 \quad (4)$$

$$\text{Yield} = -133.480 - 0.543F_1 - 44.14F_2 + 63.95F_3 + 14.0F_4 - 1.094F_5 + 0.000114F_1^2 - 7.269F_2^2 - 3.476F_3^2 - 0.398F_4^2 - 0.00312F_5^2 - 0.05701F_1F_2 + 0.01684F_1F_3 + 0.01722F_1F_4 + 0.00157F_1F_5 + 4.252F_2F_3 + 2.653F_2F_4 + 0.217F_2F_5 - 1.296F_3F_4 - 0.07325F_3F_5 + 0.01868F_4F_5 \quad (5)$$

$$\text{Efficiency} = 43.020 - 1.78F_1 - 40.27F_2 + 137.42F_3 + 1.195F_4 - 1.783F_5 + 0.000367F_1^2 - 19.27F_2^2 - 5.723F_3^2 - 0.07307F_4^2 - 0.00451F_5^2 + 0.06209F_1F_2 + 0.0075F_1F_3 + 0.04057F_1F_4 + 0.00274F_1F_5 + 10.7F_2F_3 + 3.999F_2F_4 + 0.378F_2F_5 - 3.996F_3F_4 + 0.00558F_3F_5 - 0.01093F_4F_5 \quad (6)$$

where ethanol, yield and efficiency are the ethanol concentration (g/l), ethanol yield (%), and fermentation efficiency (%), respectively. F_1, F_2, F_3, F_4, and F_5 are total sugar concentration (g/l), pH, yeast suspension (10^Z cells/ml), temperature (°C), and time (h), respectively.

Table 3. Analysis of variance of the response surface models for nipa sap fermentation.

Terms	Ethanol concentration		Ethanol yield		Fermentation efficiency	
	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value
b_0	-171.470	0.788	-133.480	0.539	43.020	0.965
b_1	-0.281	0.716	-0.5430	0.063	-1.780	0.163
b_2	-109.690	0.280	-44.140	0.204	-40.270	0.791
b_3	86.200	0.321	63.950	0.049	137.420	0.309
b_4	28.740	0.109	14.000	0.031	1.195	0.963
b_5	-3.038	0.031	-1.094	0.023	-1.783	0.350
b_{11}	< 0.001	0.678	0.0001	0.563	-0.000	0.683
b_{22}	-14.820	0.047	-7.269	0.009	-19.270	0.085
b_{33}	-7.601	0.135	-3.476	0.054	-5.723	0.443
b_{44}	-0.828	0.002	-0.398	0.000	-0.073	0.795
b_{55}	-0.006	0.002	-0.003	0.000	-0.004	0.147
b_{12}	-0.135	0.096	-0.057	0.046	0.062	0.592
b_{13}	0.045	0.490	0.017	0.444	0.008	0.940
b_{14}	0.021	0.119	0.017	0.003	0.040	0.060
b_{15}	0.004	0.003	0.002	0.002	0.003	0.138
b_{23}	12.810	0.089	4.252	0.092	10.700	0.327
b_{24}	5.312	0.003	2.653	0.000	3.999	0.077
b_{25}	0.420	0.006	0.217	0.000	0.378	0.065
b_{34}	-2.032	0.102	-1.296	0.008	-3.996	0.047
b_{35}	-0.083	0.424	-0.073	0.058	0.006	0.972
b_{45}	0.046	0.042	0.019	0.019	-0.011	0.720
R^2	0.981		0.981		0.821	
Adj R^2	0.935		0.933		0.374	
F	21.210		20.420		1.837	
F Signif	<< 0.001		<< 0.001		0.190	
Std Error	7.631		2.570		11.830	

The interaction effects of the two parameters can be explained by a three dimensional response surface with central level fixing of the other parameters. The central levels of total sugars, pH, yeast, temperature, and time, that were used to plot Figures 1, 2, and 3, were 212.6 g/l, 5.5, 10^7 cells/ml, 34 °C, and 77 h, respectively.

Figure 1 shows the parameter effects on the ethanol concentration. The ethanol concentration clearly increased as the initial sugar content increased for all process conditions (Figures 1A–1D). On the other hand, there was no improvement in ethanol concentrations with yeast suspension higher than 10^7 cells/ml (Figures 1B, 1E, 1F, and 1G), pH higher than 5.4 (Figures 1A, 1E, 1H, and 1I), and temperature higher than 34 °C (Figures 1C, 1F, 1H, and 1J). This suggests that the fermentation time can be reduced as long as the concentration of initial total sugars is high enough regardless of increased yeast suspension or fermentation temperature. However, to achieve the optimal concentration of ethanol (>100 g/l), the yeast needs to be in a temperature range of 31–34 °C with an initial pH in the range of 4.7–5.4 for a sufficient fermentation time in the range of 70–95 h.

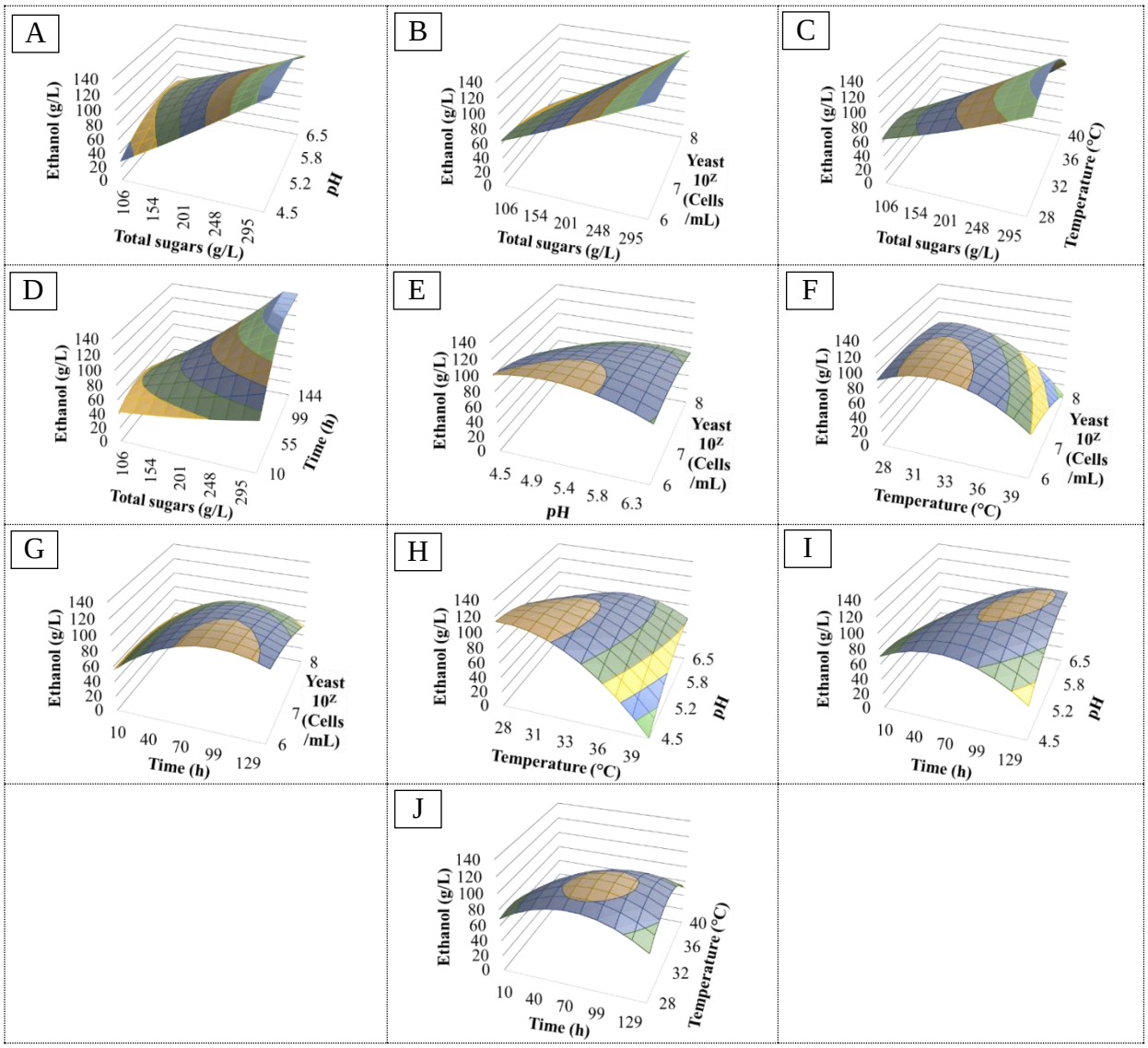

Figure 1. Ethanol concentration in nipa sap fermentation as a function of: (A) total sugar concentration and pH, (B) total sugar concentration and yeast suspension, (C) total sugar concentration and temperature, (D) total sugar concentration and time, (E) yeast suspension and pH, (F) yeast suspension and temperature, (G) yeast suspension and time, (H) pH and temperature, (I) pH and time, and (J) temperature and time.

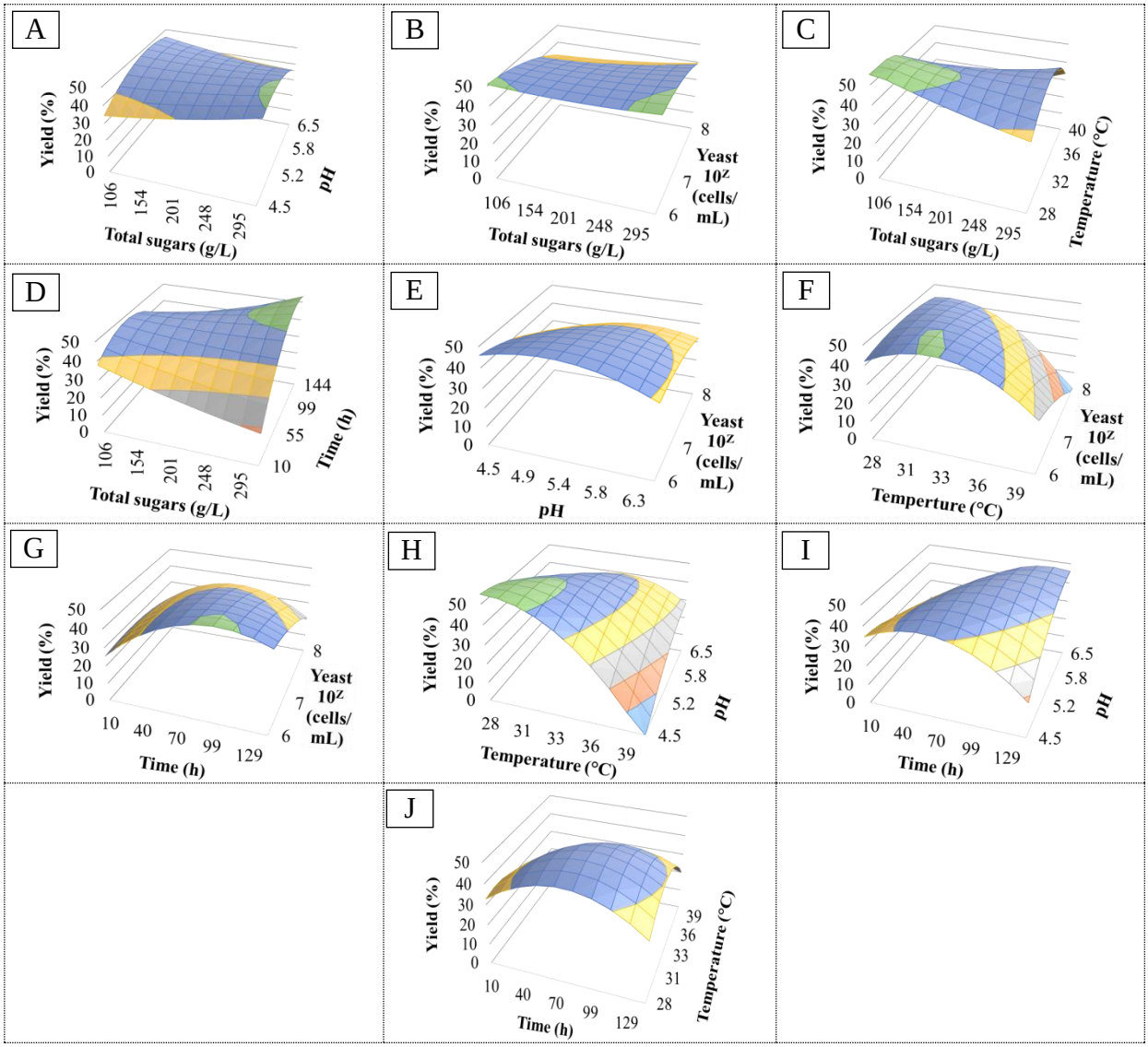

Figure 2. Ethanol yield in nipa sap fermentation as a function of: (A) total sugar concentration and pH, (B) total sugar concentration and yeast suspension, (C) total sugar concentration and temperature, (D) total sugar concentration and time, (E) yeast suspension and pH, (F) yeast suspension and temperature, (G) yeast suspension and time, (H) pH and temperature, (I) pH and time, and (J) temperature and time.

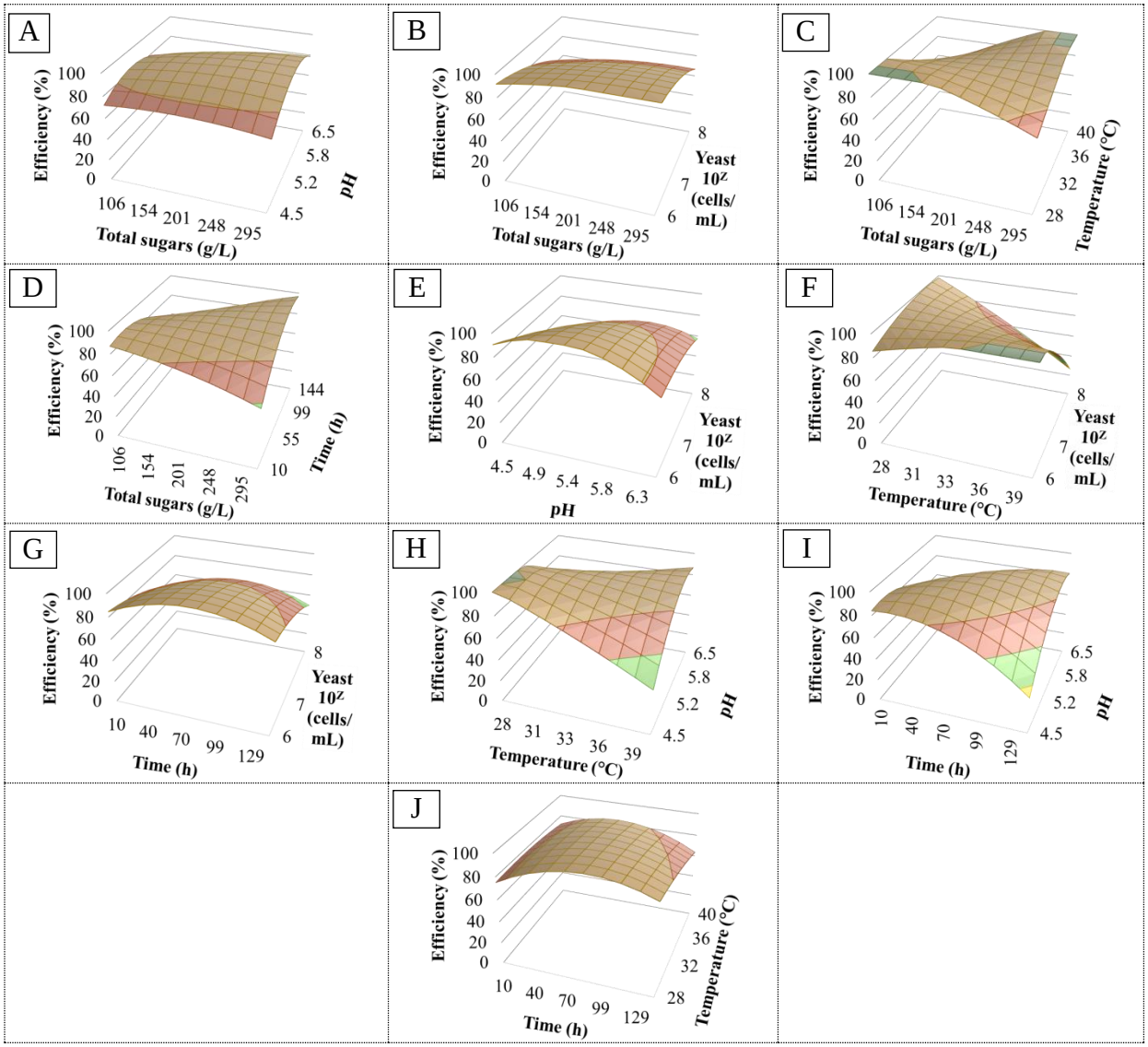

Figure 3. Fermentation efficiency in nipa sap fermentation as a function of: (A) total sugar concentration and pH, (B) total sugar concentration and yeast suspension, (C) total sugar concentration and temperature, (D) total sugar concentration and time, (E) yeast suspension and pH, (F) yeast suspension and temperature, (G) yeast suspension and time, (H) pH and temperature, (I) pH and time, and (J) temperature and time.

3.2.2 Response analysis of the ethanol yield

The polynomial model (Equation 5) which predicts the ethanol yield was developed from the data of Table 2. The coefficients of the model including a very high coefficient of determination ($R^2 = 0.981$) and the ANOVA results are given in Table 3. The *P*-values for the yield response (Table 3) showed that the significant influences on the yield were individual, quadratic, and interaction effects of time, and the results were similar for the concentration response. However, the individual effects of yeast suspension and temperature were also significant for the yield. In addition, the quadratic effects of pH and temperature were highly significant. The interaction effects of all parameters clearly influenced the yield, except the effects between total sugars and yeast, pH and yeast, as well as time and yeast. The interaction effects of the five parameters are illustrated with surface plots in Figure 2.

There was no improvement of the yield with increasing total sugars (Figures 2A–2D). The interactions between the yeast load, which ranged from 10^6 to 10^8 cells/ml, and the other parameters had no influence on the ethanol yield (Figures 2B, 2C, 2F, and 2G). The reason may be because the sap had naturally native living yeasts; therefore, the external yeast addition of 10^6 cells/ml was sufficient. On the other hand, the interactions between temperature and the other parameters highly influenced the ethanol yield. The yield decreased due to temperatures higher than 32 °C (Figures 2C), 2F, 2H, and 2J). Similarly, a further increase of time (>90 h) did not increase the yield (Figures 2G, 2I, and 2J).

3.2.3 Response analysis of the fermentation efficiency

Fermentation efficiency is a kinetic parameter that represents the potential of the yeast isolated from the nipa sap as fermenting medium. The major effect on the fermentation efficiency comes from the interaction of yeast suspension and temperature only (Table 3). This implied that temperature extremely affected the yeast function. According to the results from *P*-values, none of the operating variables were significant. The changes in the values of the variables in the ranges of this study have less effect on the efficiency considering that the fermentations proceeded in the optimal ranges of 4.5–6.5 pH at 28–40 °C for the growth and function of yeasts (Le & Le, 2014).

The effects of total sugar concentration, pH, yeast suspension, temperature, and time on the fermentation efficiency are shown in Figures 3A–3J. It was found that the efficiency decreased while increasing the concentration of total sugars at higher than 212.6 g/l (20% [w/w]) (Figures 2A–2D). This may be because a substrate with a high sugar concentration inhibits the yeast growth and fermentation to ethanol. Inhibition may be due partly to osmotic pressure. When the concentration of sugar is higher than 14% w/w, plasma cells can occur and inhibit the enzyme activity in the glycolysis (Cazetta *et al.*, 2007). The pH and temperature were environmental factors importantly affecting the yeast. The initial pH value is essential for batch fermentation without pH control. Organic acid production and carbon dioxide diluted in broth (by-product from the fermentation) cause a lower pH value and the production of ethanol may decrease with uncontrolled pH (Ergun & Mutlu, 2000). However, the ethanol production can potentially be carried out if the pH does not go lower than 4.0. A suitable initial pH depends on the type of substrate. An initial pH between 4.23 and 4.56 was appropriate for ethanol from oil palm frond juice (Srimachai *et al.*, 2015). Initial pH values of 5.1 and 5.7 were suitable for nipa leaf (Le & Le, 2014) and corncob hydrolysate (Chang *et al.*, 2012), respectively. In this work, the suitable pH was in the range of 4.7–5.4 (Figures 3A, 3E, 3H, and 3I) while the temperature should not be higher than 34 °C (Figures 3C, 3F, 3H, and 3J). These suitable ranges of pH and temperature were in the ranges for the growth of the yeast (Charoenchai *et al.*, 1998; Le & Le, 2014). In addition, a sufficient process for the ethanol production was observed within the first 80 h. Later the efficiency decreased (Figures 3G, 3I, and 3J).

3.3 Optimization of the fermentation

The five factors could be predicted by the regression models to achieve optimal conditions. The 173.4 g/l of the predicted maximum ethanol concentration would be obtained using a pH of 4.5, a total sugar concentration of 318.9 g/l, a yeast suspension of 10^6 cells/ml, a temperature of 31 °C and a fermentation period of 94 h. Meanwhile, the 53.4% optimal yield would be achieved with pH 4.8 using a total sugar concentration of 212.6 g/l and 10^6 cells/ml of yeast suspension at 32 °C for 77 h, and the 100% fermentation efficiency would be reached at a pH of 5.4 using a total sugar concentration of 212.6 g/l and 10^6 cells/ml of yeast suspension at 34 °C for 77 h. The optimal yield and efficiency were acquired under slightly different conditions, whereas the optimal concentration was very different. Only a yeast suspension of 10^6 cells/ml was optimal for the fermentation by all three responses. Evidently, the initial total sugar concentration had a great effect on the ethanol concentration but only a small effect on the yield and the efficiency. A high ethanol concentration is desirable in order to reduce energy consumption in ethanol distillation. At the same time, minimizing total ethanol production costs is as important as maximizing the ethanol yield and fermentation efficiency. In terms of usage, ethanol yield is the priority for the commercialization compared with fermentation efficiency. Under the optimal yield condition, the experimental ethanol yield was 53.2%, which provided an experimental ethanol concentration of 113.1 g/l with 1.5 g/l h ethanol productivity and 98.6% fermentation efficiency.

4. Conclusions

The results showed that nipa sap can be a potential feedstock for ethanol production using isolated yeast without nutrient supplementation in a batch fermentation process. RSM was used to build mathematical models of the nipa sap fermentation to optimize the process conditions. This data could be useful for further development. The use of the sap offers advantages over the use of sugarcane in cultivation, harvest, environmental effects, and economic efficiency. It is an alternative form of energy production along with maintaining good ecology. However, consideration must be

given on how best to preserve nipa palm forests. Further research is required to ensure the possibility of sustainable commercial production.

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