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Original Article

Nipa sap pretreatment for bioethanol fermentation

Sininart Chongkhong* and Kanokkan Srinoon

Department of Chemical Engineering, Faculty of Engineering,
Prince of Songkla University, Hat Yai, Songkhla, 90112 Thailand

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Abstract

The pretreatment of nipa sap to improve the efficiency of ethanol fermentation was investigated. The ability of the pretreatment to increase fermentable sugars and yeast cells in the sap, and the suitability of nipa sap for fermentation was assessed. Effects of pH, temperature and duration of the pretreatment were studied, and response surface models were created for reducing sugar, total sugar, and yeast cell yields. Temperature was the most important pretreatment variable, followed by pH value, for all the outputs modeled. The maximum cell yield of 829% (from 0.7×10^5 to 6.5×10^5 CFU/mL) was achieved with a pH of 5.3 at 44 °C for 21 minutes. These conditions gave a total sugar yield of 39% (from 266 to 369 g/L) and a reducing sugar yield of 422% (from 36 to 188 g/L). The fermentation of pretreated and non-pretreated saps was compared, and it was found that the pretreatment increased fermentation efficiency.

Keywords: nipa sap, pretreatment, bioethanol, microorganisms, natural yeast

1. Introduction

Bioethanol is a sustainable and environmentally friendly energy source of ongoing interest, since it can be used directly as transportation fuel or blended with gasoline. Bioethanol can be produced from three major types of feedstock, juices or molasses from sugar crops, starches from cereal crops, and lignocellulosic biomass from agricultural residues. However, both sustainability and economic viability are important for the bioethanol industries, and currently the major type of feedstock is sugar crops (Zabed *et al*., 2014) that allow relatively easy direct conversion of the free sugars to ethanol, thus avoiding the costly hydrolysis step (Abdullah *et al*., 2015; Germec *et al*., 2015; Gumienna *et al*., 2016; Luo *et al*., 2014).

Apart from sugarcane juice, an alternative sugar-rich juice in Thailand is nipa sap from the nipa palm (*Nypa fructicans*), which has some advantages over sugarcane, such as higher production of sugar (Dalibard, 1999) without the need for fertilizers, herbicides, insecticides or heavy machinery in plantations (Tamunaidu *et al*., 2013). Nipa palms are distributed throughout Asia and Oceania in coastal and estuarine habitats (Jabatan, 2009). The palms can yield 6,480-15,600 liters of fuel per year per hectare, while sugar-cane can yield 5,000-8,000 liters/year/hectare (Wikipedia, 2016). Nipa sap can be consumed as a fresh beverage, or processed into sugar, vinegar and alcohol. Nipa sap, like sugarcane juice, is an ideal raw material for bioethanol production because it contains adequate amounts of minerals, organic nutrients and microorganisms (Tamunaidu *et al*., 2013). However, these factors cause some problems with storage and "shelf-life", due to microbial decomposition (Dodic′*et al*., 2009; Lipnizki *et al*., 2006; Tamunaidu *et al*., 2013).

Prior studies on bioethanol production from juices have mainly focused on the fermentation stage, even though pretreatments are also important because they can reduce problems and increase fermentation efficiency. The selection of a suitable pretreatment depends on the nature of the raw materials. For instance, sorghum stalk juice was pretreated at 80 °C followed by the addition of phosphoric acid to remove phenolic compounds and impurities, and the application of poly-aluminum chloride (PAC) to clarify the otherwise cloudy juice. Reduction in phenolic compounds may favor yeast cell

*Corresponding author
Email address: sininart.c@psu.ac.th

growth, but the clarification negatively affects cell growth (Kartawiria *et al.*, 2015). In addition, filtration and carbonation are used to treat sugarcane juice intended for human consumption (Doherty *et al.*, 2003; Kochergin *et al.*, 2010; Pratti & Moretti, 2010).

Nipa sap, with its rich nutrients and sugars, supports the growth of microorganisms and the sap is a source of several organisms such as acid tolerant bacteria, yeast and mold. These can easily decompose the sugars and spoil the sap. These problems have been solved by using thermal processing to concentrate the sap into syrup or dry sugar (palm sugar). Thermal processing at an elevated temperature to evaporate water also destroys some nutrients and minerals, kills microorganisms including yeasts, and has energy costs. This represents the major impediment to the commercialization of nipa sap ethanol.

This study used a pretreatment method to improve the efficiency of bioethanol fermentation of nipa sap. The study clarifies the influence of environmental factors during pretreatment on the native microorganisms making useful enzymes in the sap. Enzymes are found in every living cell including in all microorganisms. For example, important hydrolyzing enzymes, including amylases from bacteria and invertase from yeast, provide reducing sugar (Underkofler *et al.*, 1958). However, in order to reduce problems and increase fermentation efficiency, thus lowering the ethanol production costs, pretreatments have to suitably support the growth and function of yeasts over other microorganisms. Therefore, this work focused on pretreating nipa sap so as to promote the natural microorganisms that increase fermentable sugars in the sap, as both sufficient nutrient sugars and active yeasts will contribute to the fermentation that follows. The pretreatment must be restricted to low temperatures (30-60°C) to retain the nutrients and to support yeast growth (Charoenchai *et al.*, 1998), and have an acidic pH in the range of 4.5-6.5 for the survival and growth of the yeasts (Le & Le, 2014). A further variable capable of manipulation is the preprocessing time (5-30 minutes). These factors were investigated and optimized by response surface methodology, and then the optimally pretreated sap was fermented to confirm the practical effects of the pretreatment on the final product.

2. Materials and Methods

2.1 Nipa sap

Nipa sap was obtained from the Chan Tarang Sri plantation, located on swampy land with flooding in the rainy season (November-January), located in Pak Phanang Basin, Nakhon Si Thammarat Province, Thailand. The sap collected early in the morning (before 7.00 a.m.) was immediately stored at 4 °C until use.The raw sap used in this study contained 36 and 266 g/L initial concentrations of reducing sugar and total sugars (sum of reducing sugar and non-reducing sugar), respectively, and 0.7×10^5 and 1.0×10^5 CFU/mL initial cells of natural yeast and total bacteria, respectively.

2.2 Pretreatment

The sap was filtered to remove impurities and 100 mL working volumes were poured into 250 mL bottles. The experimental design of the pretreatment is shown in Table 1. The pH was adjusted to the assigned initial value with sulfuric acid or sodium hydroxide solution, and then the uncapped bottles were placed in a water bath at the given temperature, for the assigned time. The pretreated sap was sampled to analyze the yeast cells, and filtered to achieve a clear liquid for the analysis of the reducing sugar and total sugars in order to determine the optimal pretreatment conditions for fermentation.

Table 1. Experimental conditions and results for nipa sap pretreatment.

Exp. No.	Conditions			Experimental results		
	pH	Temperature (°C)	Time (min)	Reducing sugar yield (%)	Total sugar yield (%)	Cell yield (%)
1	4.5	45	18	419	18	528
2	4.9	36	10	441	22	377
3	4.9	36	25	472	15	368
4	4.9	54	10	323	32	477
5	4.9	54	25	364	35	707
6	5.5	30	18	430	33	654
7	5.5	45	5	398	34	850
8	5.5	45	18	409	41	807
9	5.5	45	18	409	41	810
10	5.5	45	18	410	41	809
11	5.5	45	30	395	26	869
12	5.5	60	18	336	36	-49
13	6.1	36	10	362	35	300
14	6.1	36	25	367	33	257
15	6.1	54	10	312	21	379
16	6.1	54	25	341	26	-97
17	6.5	45	18	397	1	343

2.3 Fermentation without nutrient supplementation

Preliminary bioethanol fermentation was conducted to assess the efficiency of the sap pretreatment. The sap pretreated under the optimal conditions was cooled to room temperature, and diluted with deionized water to obtain a substrate having an initial total sugar concentration of 214 g/L (about 20 %w/w, i.e. 20 g total sugars in 100 g of substrate). The pH of the substrate was adjusted to 5.5 before fermentation. The fermentation was carried out in 250 mL conical flasks with a working volume of 100 mL substrate. The flask was sealed with a rubber septum with an air-locked tube inserted, and was placed in an incubator shaker (LabTech, LSI-3016A, South Korea) set at 30 °C and 80 rpm. Samples were collected for the analysis of ethanol concentration at various times during the batch fermentation under anaerobic conditions, which lasted a total of 60 hrs.

Fermentations were conducted with otherwise similar experimental conditions to compare three options: fermentation of pretreated sap with or without the addition of 0.1 g baker's yeast, *Saccharomyces cerevisiae*, with a cell count of 1×10^{10} CFU/g (instant yeast under the trade name Fermipan brown was used) and non-pretreated sap without added yeast.

2.4 Analytical methods

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

The aim of this study of sap preparation was to consider all the pretreatment products which are crucial to the yield. The products that support efficient fermentation are the fermentable sugars (total sugars) including reducing sugar and non-reducing sugar, and yeast cells. Consequently, the pretreatment should ideally increase both the sugars and the yeast cells for the eventual optimization of the fermentation yield.The yields of reducing sugar, total sugars and yeast cells in Equations (1) to (3), are the percentage increases in the reducing sugar concentration, the total sugar concentration and the yeast cell count, respectively and are expressed on scales such that the initial sap without pretreatment has a value of 100. The reducing sugar and total sugar yields were calculated as follows:

$$\text{Reducing sugar yield (\%)}=\frac{\text{reducing sugar concentration in pretreated sap (g/L)} - 36\ \text{(g/L)}}{36\ \text{(g/L)}} \times 100\% \quad (1)$$

$$\text{Total sugar yield (\%)} = \frac{\text{total sugar concentration in pretreated sap (g/L)} - 266\ \text{(g/L)}}{266\ \text{(g/L)}} \times 100\% \quad (2)$$

where 36 and 266 g/L are the initial concentrations of reducing sugar and total sugar before pretreatment, respectively. The yeast cell count(in colony-forming units, CFU) following FDA 2001 Bacteriological Analytical Manual, U.S. Food and Drug Administration, APHA (water) 2005, was determined by the Department of Microbiology, Faculty of Science, Prince of Songkla University. The cell yield was calculated as:

$$\text{Cell yield (\%)}=\frac{\text{cell count in pretreated sap (CFU/mL)} - 0.7 \times 10^5\text{(CFU/mL)}}{0.7 \times 10^5\text{(CFU/mL)}} \times 100\% \quad (3)$$

where 0.7×10^5 CFU/mL is the initial cell count before pre-treatment.

The ethanol concentration after fermentation was determined using gas chromatography (GC 6890, Hewlett Packard, USA) with an auto injector, HP-FFAP polyethylene glycol TPA column (column size: 0.5 µm × 0.32 mm × 25 m), and a flame ionization detector, with emphasis on the solvents, ethanol and acetone in solution. The oven temperature was held at 85 °C, the injector at 150 °C, and the detector at 250 °C. In the carrier gas, the nitrogen flow rate was set at 25 mL/min, that of hydrogen at 44.6 mL/min, and that of air at 300 mL/min. The ethanol conversion (from the sugars at the beginning to ethanol), fermentation efficiency (or conversion from the sugar used in fermentation to ethanol) and ethanol productivity were calculated as follows:

$$\text{Ethanol conversion (\%)}=\frac{\text{ethanol obtained in fermentation (g/L)}}{\langle 0.511\times\text{reducing sugar at the beginning (g/L)}\rangle + \langle 0.538\times\text{non-reducing sugar at the beginning (g/L)}\rangle} \times 100\% \quad (4)$$

$$\text{Fermentation efficiency (\%)}=\frac{\text{ethanol obtained in fermentation (g/L)}}{\langle 0.511\times\text{reducing sugar used in fermentation (g/L)}\rangle + \langle 0.538\times\text{non-reducing sugar used in fermentation (g/L)}\rangle} \times 100\% \quad (5)$$

$$\text{Ethanol productivity} = \frac{\text{ethanol obtained during fermentation (g/L)}}{\text{fermentation time (h)}} \quad (6)$$

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

2.5 Statistical analysis of the pretreatment

The central composite design (CCD) with three process factors provided 17 total experiments (14 non-repeated cases and 3 replicates of the center point), as shown in Table 1, for investigating the responses in reducing sugar, total sugar, and cell yield. The five factor levels of each manipulated variable were: initial pH at 4.5, 4.9, 5.5, 6.1 and 6.5; pretreatment temperatures 30, 36, 45, 54 and 60 °C; and pretreatment times 5, 10, 18, 25, and 30 minutes. The results were processed statistically by analysis of variance (ANOVA). The model fits were assessed from the coefficient of determination (R^2) and the model ***P***-value. A full quadratic polynomial regression model was employed to fit the experimental data:

$$Y=b_0 + b_1*pH + b_2*T + b_3*t + b_{11}*pH*pH + b_{22}*T*T + b_{33}*t*t + b_{12}*pH*T + b_{13}*pH*t + b_{23}*T*t \quad (7)$$

where Y is the response variable, i.e., one of reducing sugar yield (%), total sugar yield (%) or cell yield (%); The symbols, pH, T and t represent the pH value, the temperature (°C) and the pretreatment time (minutes), respectively. The

coefficient b_0 is the intercept, b_1, b_2 and b_3 are for the linear terms, b_{11}, b_{22} and b_{33} are for the quadratic terms, and b_{12}, b_{13} and b_{23} indicate interactions of the factors. The quadratic model equations were used to plot surfaces in Microsoft Excel 2010 to assess the individual and interaction effects of the factors on the responses, while in each such surface plot the third factor was fixed at its central level.

3. Results and Discussion

3.1 Effects of pretreatment on nipa sap

The experimental conditions and results are summarized in Table 1. These results were used to fit the quadratic polynomial models for reducing sugar yield (Equation (8)), total sugar yield (Equation (9)) and yeast cell yield (Equation (10)).

$$\text{Reducing sugar yield (\%)} = 698.370 - 8.322*pH - 8.116*T + 9.068*t - 14.930*pH*pH - 0.178*T*T - 0.171*t*t + 3.497*pH*T - 0.911*pH*t + 0.066*T*t \quad (8)$$

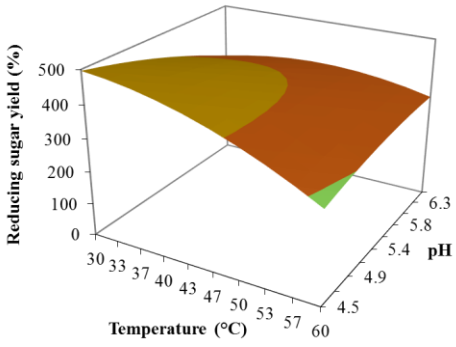
$$\text{Total sugar yield (\%)} = -1124.600 + 366.830*pH + 7.522*T - 0.941*t - 29.040*pH*pH - 0.016*T*T - 0.052*t*t - 1.184*pH*T + 0.215*pH*t + 0.032*T*t \quad (9)$$

$$\text{Cell yield (\%)} = -24331.500 + 6197.900*pH + 335.460*T + 140.520*t - 479.240*pH*pH - 2.720*T*T - 0.363*t*t - 16.540*pH*T - 20.010*pH*t - 0.449*T*t \quad (10)$$

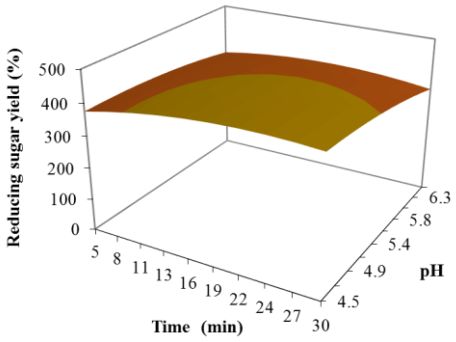
where pH, T and t are pH value, temperature (°C), and pretreatment time (minutes), respectively. Figures 1(a)-(c) show the factor effects on the reducing sugar yield. For all pH levels and pretreatment times, as shown in Figures 1(a) and (c), the reducing sugar yield decreased with temperatures from 45 to 60 °C. This suggests that the sap should be treated at low temperature (30-45 °C) for the optimal yield of reducing sugar, under an acidic pH level (4.5-4.9), see Figures 1(a) and (b), for up to 22 minutes, Figures 1(b) and (c). There was no improvement in the yield with pH levels higher than 4.9 or treatment times exceeding 22 minutes. This might be because the reducing sugar yield was obtained from microbiological pretreatment with enzymes (namely gluco-amylase and invertase), and these enzymes are effective at a pH level between 4.0 and 6.0 at temperatures between 30 and 50 °C (Montgomery & Bochmann, 2014).

However, thermal chemical pretreatment under acidic conditions might contribute more to the total sugar yield (sum of the reducing and non-reducing sugars) than to the microbiological pretreatment, as shown in Figures 2(a)-(c). The optimal yield of total sugar was achieved at temperatures in a range 45-60 °C, Figure 2(a), with a pH value in a range of 4.9-5.4, Figures 2(a) and (b), and a pretreatment time in a range of 16-22 minutes, Figures 2(b) and (c). These results agree with prior results on treatments of rice grain (Tian et al., 2006) and of sugarcane juice (Panpae *et al*., 2008) affecting the accumulation and degradation of sucrose (a non-reducing sugar), which were strongly affected by pH and temperature. Under less acidic conditions (pH>4.9), the activity of the sucrase/invertase enzymes is decreased, which slows down sucrose degradation to mono-sugars/reducing sugars including glucose and fructose. Moreover, under higher temperatures (>45 °C), the degradation of sucrose also decreased, whereas the accumulation of sucrose increased.

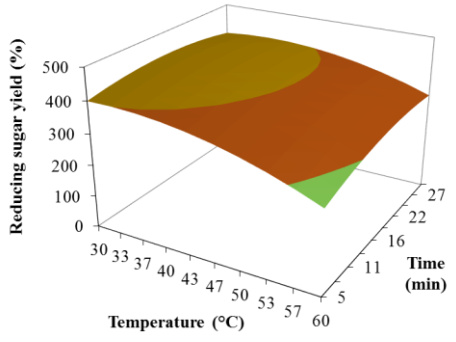
The pH, temperature and time effects on the yeast cell count are shown in Figures 3(a)-(c). The results clearly indicate that the optimal cell yield was at temperatures from 43 to 47 °C, with pH values from 4.9 to 5.4, Figures 3(a) and (b), and pretreatment times from 8 to 24 minutes, Figures 3(b) and (c). These findings agree with prior reports on appropriate pH values (4.5-6.5) and temperatures (20-50 °C) for the yeast growth (Watson, 1987; Walker, 1998).



(a)

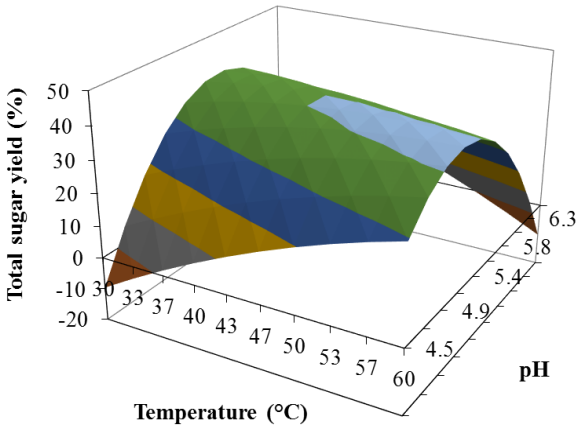


(b)

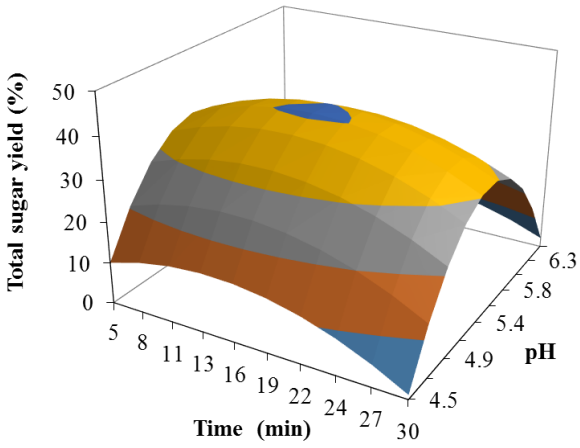


(c)

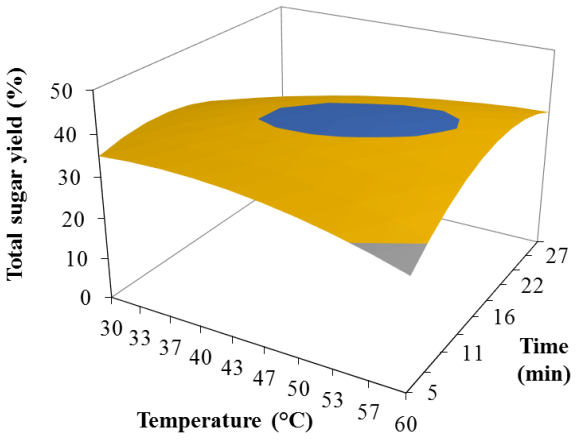
Figure 1. Reducing sugar yield in nipa sap pretreatment as a function of: (a) pH and temperature for 18 min duration, (b) pH and time at 45°C, and (c) temperature and time at pH 5.5. Plots are based on fitted response surface models.



(a)

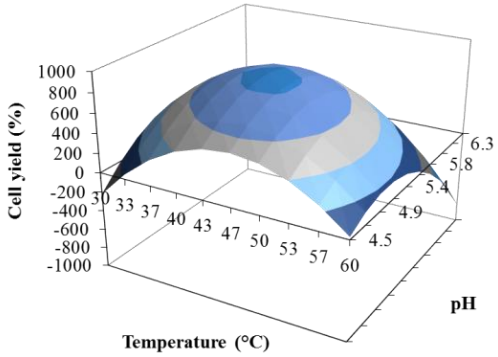


(b)

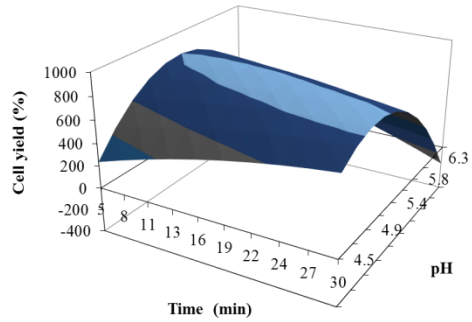


(c)

Figure 2. Total sugar yield in nipa sap pretreatment as a function of: (a) pH and temperature for 18 min duration, (b) pH and time at 45°C, and (c) temperature and time at pH 5.5. Plots are based on fitted response surface models.



(a)



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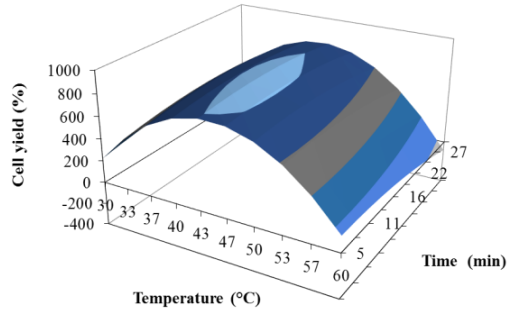

(c)

Figure 3. Yeast cell yield in nipa sap pretreatment as a function of: (a) pH and temperature for 18 min duration, (b) pH and time at 45°C, and (c) temperature and time at pH 5.5. Plots are based on fitted response surface models.

The results of the analysis of variance (ANOVA) for the models of reducing sugar yield, total sugar yield and cell yield are shown in Table 2. A probability (*P*-value) below 0.05 indicates that the individual, quadratic and interaction effects of variables are significant (the significance of the model terms). From the result, it can be seen that all the manipulated process variables (pH, temperature and time) significantly affected the total sugar yield, while the probability levels were less consistent for effects on cell yield and reducing sugar yield. In Table 2, the major effects on the reducing sugar yield came from the quadratic effect of

Table 2. Analysis of variance of the response surface models for nipa sap pretreatment.

Terms	Reducing sugar yield		Total sugar yield		Cell yield	
	Coefficient	*P*-value	Coefficient	*P*-value	Coefficient	*P*-value
b_0	698.370	0.413	-1124.600	< 0.001	-24331.500	0.015
b_1	-8.322	0.972	366.830	< 0.001	6197.900	0.024
b_2	-8.116	0.514	7.522	0.026	335.460	0.020
b_3	9.068	0.489	-0.941	0.747	140.520	0.269
b_{11}	-14.930	0.474	-29.040	< 0.001	-479.240	0.037
b_{22}	-0.178	0.081	-0.016	0.442	-2.720	0.013
b_{33}	-0.171	0.219	-0.052	0.113	-0.363	0.770
b_{12}	3.497	0.058	-1.184	0.012	-16.540	0.293
b_{13}	-0.911	0.637	0.215	0.622	-20.010	0.289
b_{23}	0.066	0.611	0.032	0.294	-0.449	0.711
R^2	0.875		0.893		0.760	
Adj R^2	0.713		0.756		0.452	
F	5.423		6.495		2.468	
F Signif	0.018		0.011		0.123	
Std Error	23.550		5.327		221.980	

temperature and its interaction with pH. The total sugar yield was dominated by the linear effects of the pH and temperature, the quadratic effect of the pH, and the interaction of pH and temperature. The cell yield had significant linear effects from the pH and temperature and quadratic effects from the pH and temperature, even though the overall model was not significant based on the *P* value which exceeded 0.05.

The *P*-values shown in Table 2 provide an overview of the pretreatment responses (yields of reducing sugar, total sugar, and yeast cells) and indicate that temperature was the crucial factor in the nipa sap pretreatment, followed by pH, while time was not significant within the range covered experimentally. Similarly, as can be seen in Figures 1 – 3, both pH and temperature had important effects on the responses, and temperature was the dominant factor in all the responses followed by pH which together influenced the total sugar and cell yields. These results confirm that pH and temperature are significant determinants of the accumulation of sugars, including reducing and non-reducing sugars, (Panpae *et al*., 2008; Tian *et al*., 2006), and of the growth of yeast cells (Walker, 1998; Watson, 1987).

Certainly, temperature and pH are important environmental factors in the survival, growth and functioning of yeast. While the yeast activity causes a change in pH, that may lead to a pH level inhibiting its own function, so it is necessary to determine the optimum time duration while maintaining the pH level in the optimal range under an assigned temperature for the desired yeast activity. Although the time effect is not statistically significant for the models, it is necessary to know what represents a sufficient time in the production process. A reduction model eliminating the insignificant model terms may therefore not be useful since the full models are necessary for determining the optimum conditions for practical production.

In addition, the pretreatment conditions were investigated by varying the controllable factors (pH, temperature and time) which might improve the functioning of yeast over other microorganisms, since the other microorganisms also interact with the yeast function during pretreatment. However, the R^2 value (Table 2) falls within the normally accepted range for an acceptable fit of the model to the data although the adjusted R^2 is somewhat outside of an acceptable range. Moreover, this study accomplishes the goal of determining if pretreatment is able to sufficiently increase the fermentable sugars and yeast cells and improve the efficiency of subsequent fermentation. Further, the pretreatment models used to establish the surface plots can also show the trends and interactions of the variables affecting the pretreatment process and provide useful information on which future development of the process can be based.

3.2 Optimization of nipa sap pretreatment

From the polynomial model Equations (8)-(10), the maximal yield of reducing sugar (497%) is achieved at a pH of 4.5 at 30 °C for 20 minutes, while the maximal total sugar yield (42%) is obtained with a pH of 5.2 at 60 °C for 20 minutes. The maximal cell yield (829%) is achieved with a pH of 5.3 at 44 °C for 21 minutes. These maximal values of the three responses clearly require different temperatures, and the pH also directly affects the yeast cell yield. Only the duration of the sap pretreatment of approximately 20 minutes is close to the optimal value for all three criteria.

All the responses, namely yields of reducing sugar, total sugars and yeast cells, are crucial criteria for the pretreatment. The reducing sugar can be directly fermented forming ethanol, the non-reducing sugar (total sugars minus reducing sugar) can be hydrolyzed by invertase or sucrase (digestive enzymes from yeast) into reducing sugar before fermenting to ethanol, and the amount of active yeast cells indicates the capacity to perform this ethanol fermentation. However, in this research the sap was pretreated as a fermentation substrate carrying the inoculum, so the survival, growth and metabolism of the yeast are of great importance. The optimal pretreatment conditions were therefore selected based on all three maximal yields, in the growth and metabolism range of the yeast. Consequently, the optimal conditions for cell yield were prioritized, and used in the current study for sap pretreatment

before fermentation. These were a pH of 5.3 at 44 °C for 21 minutes. These conditions are suitable both for the growth of the yeast (*Saccharomyces cerevisiae*) and for the function of the yeast enzymes α-amylase, α-glucosidase, maltase and glucoinvertase (pH 5.0-6.0 at 40-50 °C) (Gascon *et al*., 1968; Hostinova, 2002; Dichinson & Kruckeberg, 2006). Of course this seeming coincidence is to be expected, as sufficient growth of the yeast necessarily requires that it can effectively digest its nutrients and further improve fermentation.

The pretreatment increased the sugar content in the sap, which indicates that dissolved starch, di-/poly-saccharides and non-reducing sugar in the sap were hydrolyzed by enzymes.The sap is a source of several organisms such as acid tolerant bacteria, yeast and mold. The bacteria types detected are the acetic acid bacteria, Acetobacter, which converts ethanol to acetic acid and the lactic acid bacteria, Enterobacter, which converts glucose to lactic acid. These two types of bacteria were determined using an analysis of the total bacterial count (conducted by the Department of Microbiology, Faculty of Science, Prince of Songkla University). Although some functions of the bacteria hinder fermentation, their enzymes promote the increase in the sugar content during pretreatment. Thus, this work focused on pretreating nipa sap, to promote the natural microorganisms. However, in order to reduce the decomposition and spoilage problems, and to increase the fermentation efficiency and lower the ethanol production costs, the pretreatment has to suitably support the growth and function of yeasts which increase fermentable sugars in the sap, over other microorganisms, as both sufficient nutrient sugars and active yeasts will contribute to the fermentation that follows.

Optimal pretreatment is able to support the growth and function of yeasts over other microorganisms because it increases the yeast growth more than the total bacteria growth, while producing sufficient fermentable sugars. Pretreatment under the conditions for maximal cell yield (pH: 5.3 at 44 °C for 21 minutes) was able to increase the total sugar concentration by 39% (from 266 to 369 g/L), the reducing sugar concentration by 422% (from 36 to 188 g/L), and the yeast cells by 829% (from 0.7×10^5 to 6.5×10^5 CFU/mL), while the total bacteria cells increased by 70% (from 1.0×10^5 to 1.7×10^5 CFU/mL).

3.3 Fermentation

The pretreated sap was diluted to adjust the initial total sugar concentration to 214 g/L, which included 106 g/L reducing sugar, before fermentation. The dissolution of the nutrients, enzymatic activity and contamination can be controlled by the pH level (Liu & Shen, 2008), so pH is a crucial parameter for the fermentation. Generally the optimal pH level is between 5.0 and 5.5, whereas a pH below 4.0 causes inhibition (Grahovac *et al*., 2012). In this study, the pH of the diluted-pretreated sap was initially about 5.3 and was adjusted to 5.5. This may be a nearly optimal initial pH because the average observed final pH level was 4.3 (Table 3) and in all cases remained above 4.0. In addition, the fermentation temperature, which is another crucial parameter, was controlled at 30 °C, the temperature usually employed in culturing yeast (Alegre *et al*., 2003).

The batch fermentations were carried out with otherwise similar conditions on different substrates, namely non-pretreated sap without added yeast, and pretreated saps with and without added yeast. The production of ethanol resulting from the different substrates is shown in Figure 4 and Table 3. As can be observed in Figure 4, at the beginning

Table 3. Fermentation kinetics summary.

Fermentation time (h)	Ethanol conversion (%)	Fermentation efficiency (%)	Ethanol productivity (g/L.h)	pH
No pretreatment and fermentation without added yeast				
0	0.00	0.00	0.00	5.5
6	2.67 ± 2.03	49.23 ± 15.81	1.71 ± 0.16	4.2
12	11.82 ± 2.19	31.56 ± 5.84	1.71 ± 0.20	4.0
24	16.04 ± 1.74	32.00 ± 3.47	1.05 ± 0.08	3.8
36	32.22 ± 1.66	55.67 ± 2.87	1.21 ± 0.05	4.2
48	38.55 ± 1.79	48.39 ± 2.24	1.05 ± 0.04	5.0
60	35.74 ± 3.03	44.16 ± 3.74	0.79 ± 0.06	4.5
Pretreatment and fermentation without added yeast				
0	0.00	0.00	0.00	5.5
6	3.45 ± 0.48	52.34 ± 7.23	1.76 ± 0.09	4.4
12	36.23 ± 0.61	70.51 ± 1.18	3.95 ± 0.06	4.1
24	50.30 ± 0.68	79.23 ± 1.07	2.63 ± 0.03	4.2
36	57.06 ± 0.71	71.44 ± 0.89	1.96 ± 0.02	4.3
48	68.17 ± 0.72	83.90 ± 0.88	1.73 ± 0.02	4.5
60	68.52 ± 0.84	84.28 ± 1.03	1.39 ± 0.02	4.4
Pretreatment and fermentation with added yeast				
0	0.00	0.00	0.00	5.5
6	4.36 ± 0.47	69.95 ± 7.52	1.93 ± 0.09	4.5
12	40.45 ± 0.47	79.52 ± 0.93	4.34 ± 0.04	4.4
24	58.74 ± 0.49	93.09 ± 0.78	3.02 ± 0.02	4.3
36	65.78 ± 0.51	82.31 ± 0.63	2.24 ± 0.02	4.4
48	70.63 ± 0.53	86.30 ± 0.64	1.79 ± 0.01	4.5
60	71.34 ± 0.53	86.62 ± 0.64	1.45 ± 0.01	4.5

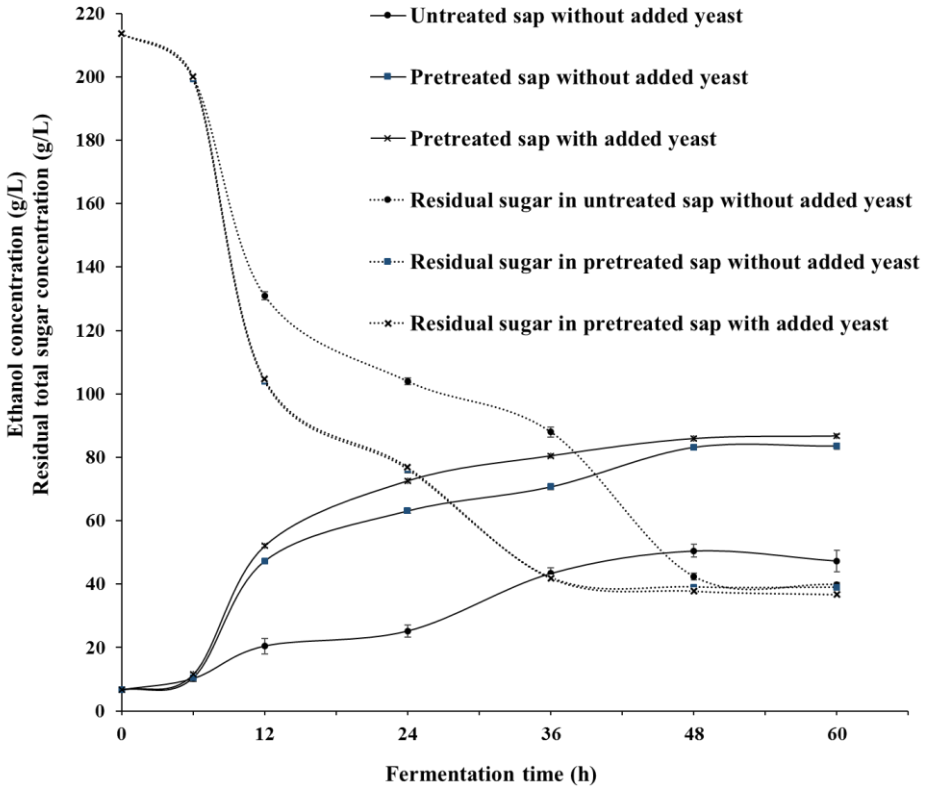

Figure 4. Ethanol fermentation of untreated sap without added yeast, pretreated sap without added yeast, and pretreated sap with added yeast, from initial pH 5.5 at 30°C.

of the fermentation, the yeast cells start adapting to the sap conditions, the yeast cells use the sugars and nutrients present, and manufacture enzymes and other attributes they need to adjust to the environment. Normally, yeast should progress through the adaptation phase and begin primary fermentation within 12 hours (Palmer, 2015). Once the cell walls of the yeast are ready to become permeable, it can start metabolizing the sugars in the sap as food, using anaerobic metabolism that converts sugars to ethanol (fermentation). The adaptation phase was similar for all three substrates, in a range of 0-6 hours with little change in total sugars. However, the ethanol concentration after 6 hours was higher in the pretreated saps than in the untreated sap. The trend in ethanol concentration was similar for the pretreated sap with and without added yeast. With pretreated media, the ethanol formation was rapid in the first 24 hours and almost complete after 48 hours, reaching ethanol concentrations of 83.2 and 85.9 g/L without and with added yeast, respectively. On the other hand, the ethanol formation with non-pretreated sap was slow and reached the maximum concentration after 48 hours (50.5 g/L), following which the concentration declined, possibly because of the production of organic acids.

Although the initial total sugar concentrations in both the pretreated sap and the non-pretreated sap were similar at 214 g/L, and the conditions (pH, temperature and time) for the ethanol fermentation were also similar, the pretreated sap contained more native yeast cells than the non-pretreated sap. This might have caused the higher ethanol yield from the pretreated sap

Based on the preliminary fermentation of the pretreated sap with added yeast for a period of 48 hours, the experimentally achieved ethanol content was 85.9 g/L (or 0.4014 g/g, ethanol to sugar), which exceeds the 0.361 g/g reported in previous work with raw nipa sap collected from a similar plantation site (Tamunaidu *et al*., 2013).

The maximum ethanol concentration was 86.7 g/L with 71.3% conversion, 86.6% fermentation efficiency and 1.4 g/L.h ethanol productivity, obtained by the fermentation of pretreated sap with added yeast for a period of 60 hours, while the pretreated sap without added yeast produced a maximum concentration of 83.6 g/L with 68.5% conversion corresponding to 84.3% efficiency and 1.4 g/L.h productivity (Table 3). These results confirm that the pretreatment improved ethanol fermentation. Even if the fermentation of nipa sap did not require external nutrients, external yeast should be added to increase the fermentation rate and efficiency in a commercial operation.

Furthermore, any residual sugars after fermentation can be dealt with in later process stages, or the fermentation of pretreated sap could be adjusted by varying the fermentation factors, including the initial sugar concentration, pH, amount of yeast, temperature, and time. It is likely that the ethanol yield, conversion and efficiency can be further improved by more comprehensive experiments.

4. Conclusions

The experimental results show that pretreatment can improve the fermentation of nipa sap by yeast to produce ethanol. The pretreatment increased both ethanol concentration and conversion along with fermentation productivity and efficiency. It was found that the sap could be fermented without nutrient supplementation and still reach high ethanol conversions, both with and without the addition of external yeast. Consequently, simple and economic ethanol production from pretreated sap might be competitive with production from conventional feedstock, including sugarcane juice and cassava starch.

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