Efficient Production of Acetic Acid from Nipa (*Nypa fruticans*) Sap by *Moorella thermoacetica* (f. *Clostridium thermoaceticum*)

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Abstract: To valorize the underutilized nipa sap composed mainly of sucrose, glucose and fructose, acetic acid fermentation by *Moorella thermoacetica* was explored. Given that *M. thermoacetica* cannot directly metabolize sucrose, we evaluated various catalysts for the hydrolysis of this material. Oxalic acid and invertase exhibited high levels of activity towards the hydrolysis of the sucrose in nipa sap to glucose and fructose. Although these two methods consumed similar levels of energy for the hydrolysis of sucrose, oxalic acid was found to be more cost-effective. Nipa saps hydrolyzed by these two catalysts were also fermented by *M. thermoacetica*. The results revealed that the two hydrolyzed sap mixtures gave 10.0 g/L of acetic acid from the 10.2 g/L of substrate sugars in nipa sap. Notably, the results showed that the oxalic acid catalyst was also fermented to acetic acid, which avoided the need to remove the catalyst from the product stream. Taken together, these results show that oxalic acid hydrolysis is superior to enzymatic hydrolysis for the pretreatment of nipa sap. The acetic acid yield achieved in this study corresponds to a conversion efficiency of 98%, which is about 3.6 times higher than that achieved using the traditional methods. The process developed in this study therefore has high potential as a green biorefinery process for the efficient conversion of sucrose-containing nipa sap to bio-derived acetic acid.

Keywords: Sucrose hydrolysis, Oxalic acid, Invertase, Acetic acid fermentation, Process simulation, Biorefinery.

1. INTRODUCTION

Nipa (*Nypa fruticans*) is a high sugar-yielding palm that can be found along coastal areas, river estuaries and mangrove forests with brackish water environments [1]. By removing the infructescence of this plant, it is possible to collect a sugar-rich sap from its stalk on a daily basis for a minimum of 60 days in the Philippines and up to 340 days in Malaysia [2]. Furthermore, nipa sap production ranges from 0.5 to 2.5 L/palm/day with an average sugar content of 16.4 w/v% [3,4].

According to Tamunaidu *et al.* [5], the major components of nipa sap are sucrose, glucose and fructose [6], which can be used for different purposes. For instance, fresh tapped nipa sap is consumed as a popular sweet drink in the coastal areas of Southeast Asia [7]. Additionally, some villages in Thailand earn a living from tapping and selling nipa sap for wine and sugar production [7]. In the Philippines, nipa sap is poured into huge earthen jars, where it is kept for up to a month for acetous fermentation to produce vinegar [8]. Despite its high potential as a source of raw materials, nipa palm is tapped limitedly by local communities for domestic use with no reported international trade [9].

Nipa is an abundant plant material in Asia, where it is regarded as a non-threatened and underutilized sugar-yielding palm [10]. It is noteworthy that nipa sap was recently assessed as a high potential feedstock for bioethanol production [11]. In fact, estimated annual ethanol yield of 3,600–22,400 L/ha/year from nipa sap [12] makes it an attractive raw material for ethanol production compared to sugarcane and corn, which afford yields of 5,300–6,500 and 3,100–3,900 L/ha/year, respectively [13]. Nipa sap could therefore become a promising source of sugars for manufacturing bioproducts.

Acetic acid is one of the most important industrial reagents in the world. Acetic acid has a broad range of applications, including its use as building block for the synthesis of monomeric vinyl acetate, ethyl acetate, butyl acetate and acetic anhydride, as well as its use as a solvent for the production of purified terephthalic acid [14,15]. However, acetic acid is mainly produced from petrochemical resources via methanol carbonylation and the liquid-phase oxidation of butane, naphtha and acetaldehyde [16]. In light of dwindling fossil fuel supplies and the push for new environmentally manufacturing processes based on renewable resources, there has been considerable interest in the production of acetic acid via bio-based routes [15].

Traditional processes for the production of vinegar from nipa sap involve a two-stage fermentation process in which alcohol is initially formed by yeast and subsequently converted to acetic acid by aerobic bacteria

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[17,18]. As shown in equation (1), two moles of carbon are lost during this process in the form of CO_2 , leading to low carbon conversion efficiency.

In contrast, among the acetogenic bacteria, *Moorella thermoacetica* can directly convert glucose and fructose to acetic acid as a single product in a stoichiometric manner [19,20], according to the equation provided below.

Notably, this fermentation process does not emit any CO_2 , and can therefore achieve a higher conversion efficiency than the traditional process described above.

Although *M. thermoacetica* has been investigated extensively for the conversion of model compounds [21–24], it has not been investigated for its conversion of natural sugar sources such as nipa sap because it contains non-fermentable sucrose [19,21]. With this in mind, the aim of the current study was to evaluate the potential use of nipa sap for the efficient bio-derived production of acetic acid.

2. MATERIALS AND METHODS

2.1. Materials

The nipa sap used in this study was collected from Sarawak in Malaysia. For conservation and transportation purposes, the sap was concentrated by heating to give a viscous liquid. Prior to the experiments described in this study, 200 g of concentrated nipa sap was diluted with deionized water to a total volume of 1 L, which gave a sugar concentration similar to that of the original sap. Invertase solution (EC No.3.2.1.26) with a minimal activity of 4 units/mL was purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Freeze-dried cultures of *M. thermoacetica*, which is also known as *Clostridium thermoaceticum* (ATCC 39073), were obtained from the American Type Culture Collection (Manassas, VA, USA).

2.2. Acid Hydrolysis

Three different acids, including acetic acid, oxalic acid and hydrochloric acid were evaluated as catalysts for the hydrolysis of sucrose in the pretreatment step.

For the hydrolysis reactions conducted with acetic acid, 0–20 g/L of the acid catalyst was added to the nipa sap in a 20 mL vial, and the resulting mixture was heated in an autoclave at 121 °C for various reaction times. The reaction mixtures were then removed from the autoclave and rapidly chilled in a refrigerator to room temperature before being neutralized ($\text{pH } 7.0 \pm 0.1$) by the addition of an appropriate buffer solution. The hydrolysis of sucrose in nipa sap was conducted in a similar manner with 1.5–4.5 g/L oxalic acid and 3.0 g/L hydrochloric acid.

2.3. Enzymatic Hydrolysis

The enzymatic hydrolyses were performed in a metal bath at 25 and 60 °C with a 3.92 v/v% invertase solution. The reactions were subsequently treated with boiling water for 5 min to destroy the invertase activity.

2.4. Acetic Acid Fermentation

The revival of *M. thermoacetica* from a freeze-dried state and the subsequent preparation of the corresponding inoculum were conducted according to the procedures reported by Nakamura *et al.* [22].

M. thermoacetica was used to produce acetic acid in a pH-controlled batch fermentation process, which was conducted in a 500 mL DPC-2A fermentation system (Able Corporation, Tokyo, Japan) at 60 °C with a stirring rate of 300 rpm. The nutrients in the broth were similar to those used by Rabemanolontsoa *et al.* [25], and consisted of the following chemicals: 5 g/L yeast extract, 0.1 g/L cysteine·HCl·H₂O, 1 g/L $(\text{NH}_4)_2\text{SO}_4$, 0.25 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g/L $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$, 0.24 mg/L $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.29 mg/L $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.017 mg/L Na_2SeO_3 and 1 mg/L resazurin. The nutrients were weighed and dissolved in 166 mL of Milli-Q water. The fermentation systems and nutrient solutions were autoclaved at 121 °C for 20 min to allow for their sterilization. An anaerobic environment was then prepared by filling a glove box with N_2 gas and a working volume of 200 mL was created in each of the fermentation systems by pouring 14 mL of hydrolyzed nipa sap, 20 mL of *M. thermoacetica* inoculum and 166 mL of nutrient solution into each fermentation system. The fermentation process conducted under an atmosphere of N_2 was maintained at $\text{pH } 6.5 \pm 0.1$ by automatic titration with 2 N NaOH. Samples were collected from the fermentation broth at specific time points through a sampling port with a sterile syringe, and then stored at –31 °C prior to being analyzed by high-performance liquid chromatography (HPLC) on an LC-10A HPLC

system (Shimadzu, Kyoto, Japan). Blank samples consisting of Milli-Q water and the catalysts instead of the hydrolyzed nipa sap were also run under the same conditions to correct for the acetic acid produced by the nutrients, inocula and catalysts.

2.5. Analyses

The concentrations of the individual sugars and ethanol in the samples were analyzed by HPLC using a Shodex sugar KS-801 column (Showa Denko, Kanagawa, Japan). The column was eluted with water at a flow rate of 1.0 mL/min with a column temperature of 80 °C. The concentrations of the organic acids present in the samples were determined by HPLC analysis over an Aminex HPX-87H column (Bio-rad, Hercules, CA, USA), which was eluted with a 5 mM aqueous H₂SO₄ solution with a flow rate of 0.6 mL/min at a column temperature of 45 °C. The inorganic elements in the nipa sap were analyzed by ion chromatography (IC) and inductively coupled plasma mass spectrometry (ICP-MS) at AU Techno Services Co., Ltd. (Osaka, Japan).

To evaluate hydrolysis efficiency, sucrose conversion to glucose and fructose and the glucose and fructose yield were estimated using the following equations:

$$\text{Sucrose conversion (\%)} = \frac{\text{Hydrolyzed sucrose (g/L)}}{\text{Initial sucrose (g/L)}} \times 100 \quad (3)$$

$$\text{Glucose and fructose yield (\%)} = \frac{\text{Total glucose and fructose produced (g/L)}}{\text{Total theoretical max. glucose and fructose produced (g/L)}} \times 100 \quad (4)$$

2.6. Process Simulation

The process responsible for the production of acetic acid from nipa sap was simulated using version 9.3 of the Pro/IITM software with a nipa sap fed rate of 750 kg/h, as shown in Figure 1. This simulation was designed as a two-stage process, including (i) the acid or enzymatic hydrolysis of sucrose; and (ii) the fermentation of acetic acid.

For the acid hydrolysis reactions, nipa sap was mixed with one of three acids, and the resulting mixture was fed into an autoclave operating at 121 °C for simultaneous hydrolysis and sterilization processes. For the enzymatic treatment process, the hydrolysis and sterilization treatments were conducted separately because they required different operating temperatures. To save energy, the stream derived from the hydrolyzed nipa sap was used to heat the untreated nipa sap prior to it being sent to the autoclave. After the hydrolysis reaction, the hydrolyzed nipa sap was depressurized to atmospheric pressure and fed together with nutrients and an aqueous NaOH solution into a fermentation system at 60 °C and 101 kPa. The aqueous nutrient and NaOH solutions were preheated by the product from the fermentation system to save energy.

Figure 1: Schematic diagrams of (a) acid hydrolysis, (b) enzymatic hydrolysis, and the subsequent (c) acetic acid fermentation processes.

The non-random two-liquid (NRTL) model was selected as the best model for the thermodynamic calculations conducted in the current study. Energy losses from the pipes and the individual units were neglected and the pump efficiency was set at 80%. Furthermore, the counter-flow heat exchangers were configured to operate with a minimum internal temperature approach ($\Delta T_{\min}$) of 10 °C.

3. RESULTS AND DISCUSSION

3.1. Chemical Composition of Nipa Sap

The chemical composition of nipa sap from Malaysia is shown in Table 1. The results in the table show that Malaysian nipa sap is predominantly composed of sucrose (78.4 g/L), glucose (32.3 g/L) and fructose (31.4 g/L). Malaysian nipa sap also contains 1.7 g/L of ethanol. The lactic acid found in this nipa sap accounts for 2.1 g/L of its composition and undoubtedly makes a significant contribution to its acidic pH (4.37). According to Nur Aimi *et al.* [26], the sugars found in nipa sap can be gradually fermented to various products, such as ethanol, lactic acid and/or acetic acid, by several naturally occurring yeasts and bacteria [11]. However, acetic acid was not detected in this case.

Table 1: Chemical Composition of Malaysian Nipa Sap

Chemical Composition	Concentration (g/L)	Content (wt%)
Organic Compounds		
Sucrose	78.4	7.31
Glucose	32.3	3.01
Fructose	31.4	2.93
Ethanol	1.7	0.16
Lactic acid	2.1	0.20
Total organics	145.9	13.61
Inorganic Elements		
K	2.6	0.24
Cl	2.6	0.24
Na	0.9	0.08
P	6.2×10^{-2}	5.8×10^{-3}
S	5.3×10^{-2}	4.9×10^{-3}
Mg	4.9×10^{-2}	4.6×10^{-3}
Ca	7.0×10^{-3}	6.5×10^{-4}
Mn	5.1×10^{-4}	4.8×10^{-5}
Al	4.9×10^{-4}	4.6×10^{-5}
Total inorganics	6.3	0.59
Nipa sap	152.2	14.20

The total inorganic content of Malaysian nipa sap was determined to be 6.3 g/L, with Na, K and Cl identified as the major elements. A trace amount of Mn was also found in this sap. This result contrasts with those of several previous studies reported by Tamunaidu *et al.* [3,11,27], where no heavy metals were detected in the sap or any other parts of the nipa palm including the frond, shell, husk and leaves.

Several other palm saps have been analyzed to date and found to contain similar quantities of inorganic constituents to those found in nipa sap, such as the Na, Mg, K and P contents in saps of palmyra, coconut and date palms [28]; the K, Mg, Ca, Na and P contents in sap of *R. hookeri* palm [29]; and the Cl, Ca, Mg, Na, Mn and P contents in sap of oil palm trunk [30].

The concentrations of Mg and Ca in nipa sap were 0.049 and 0.007 g/L, respectively. These values were similar to those found in palmyra, coconut and date palm saps, which are 0.051, 0.022 and 0.030 g/L for Mg and 0.011, 0.016 and 0.013 g/L for Ca, respectively [28]. However, nipa sap has uniquely high Na and Cl contents of 0.9 and 2.6 g/L, whereas all of the other palm saps contain only 0.043–0.054 g/L of Na and no Cl.

Although the inorganic elements found in sugarcane sap [3] are also present in nipa sap, the latter has much higher Na and Cl contents. These results therefore explain the high tolerance of nipa palm to seawater salts (e.g., NaCl) [31], and highlight nipa palm as the species of choice for seawater agriculture to initiate coastal rehabilitation and restore degraded wetlands [11].

3.2. Acid Hydrolysis

Autoclaving at 121 °C is the preferred method for sterilizing nipa sap and preventing contamination during the acetic acid fermentation process. Autoclaving can destroy all organisms and their endospores within 15–20 min [32]. According to Steven *et al.* [33], the high temperatures used in an autoclave can result in the hydrolysis of sucrose, the extent of which is directly proportional to the hydrogen ion concentration (pH). The combination of sucrose hydrolysis and sterilization with the acid hydrolysis of nipa sap was therefore investigated in an autoclave at 121 °C.

We investigated the addition of an acid catalyst to facilitate the hydrolysis of the sucrose present in nipa sap. We initially evaluated the use of acetic acid as a catalyst for the hydrolysis of sucrose because this acid

is the desired product of the anaerobic fermentation of nipa sap by *M. thermoacetica*. In this way, it was envisaged that the use of acetic acid as a catalyst would avoid the need to add and subsequently remove another chemical to the formation process and product stream, respectively.

Figure 2 shows the sucrose conversions achieved using a variety of different acetic acid concentrations as a function of time. When the hydrolysis was performed without a catalyst (Figure 2a), the conversion of sucrose in nipa sap reached 86% within 120 min. As shown in Table 1, the lactic acid present in nipa sap may account for its acidic pH and could also act as an acid catalyst for the hydrolysis of sucrose. Although the different components of nipa sap could exhibit catalytic activity towards the hydrolysis of sucrose, it was not possible to achieve the complete hydrolysis of sucrose under these conditions after 120 min. The hydrolysis of more than 98% of the sucrose in nipa sap (corresponding to a concentration of 78.4 g/L) required a treatment time of at least 120 or 60 min with 3.0–5.0 or 10–20 g/L acetic acid, respectively. The sucrose conversion improved with increasing acetic acid concentration. However, the use of a high concentration of acetic acid could inhibit the downstream fermentation of nipa sap by *M. thermoacetica* [34], and the use a prolonged hydrolysis reaction would require much more energy for autoclaving. Hence, the use of a stronger organic acid was explored to improve the efficiency of this process.

Oxalic acid is one of the strongest of all the known organic acids, with pKa values of 1.27 and 4.28 [35]. Based on its strong acidity, oxalic acid has recently been suggested as an alternative to mineral acids such as sulfuric acid and hydrochloric acid for the hydrolysis of lignocellulosic biomass because it shows a higher catalytic efficiency [36,37]. With this in mind, we investigated the use of oxalic acid as catalyst for this process.

Figure 2b shows the sucrose conversions achieved for the hydrolysis of nipa sap using various oxalic acid concentrations. The results revealed that 3.0 g/L of oxalic acid could hydrolyze 98% of the sucrose in nipa sap within 20 min, corresponding to the time required for the complete sterilization of nipa sap. These hydrolysis conditions are therefore very practical. The results also showed that the speed of the sucrose conversion increased with the augmentation of the oxalic acid concentration, which can be explained in terms of the associated decrease in the pH of the nipa sap, as shown in Table 2. Based on these results,

oxalic acid was selected as the optimum catalyst for the hydrolysis of sucrose because it allowed for a considerable decrease in the treatment time.

Figure 2: Hydrolysis of the sucrose in nipa sap to glucose and fructose at (a) 121 °C in an autoclave under various acetic acid concentrations (0, 3.0, 10 and 20 g/L); (b) 121 °C in an autoclave under various oxalic acid concentrations (0, 1.5, 3.0 and 4.5 g/L); and (c) 25 and 60 °C using 3.92 v/v% invertase.

We also investigated the hydrolysis of the sucrose in nipa sap with hydrochloric acid, a strong mineral acid, and compared the results with those of acetic acid and oxalic acid. As shown in Table 2, hydrochloric acid showed a higher level of activity towards the hydrolysis of sucrose compared with oxalic acid and acetic acid. However, the glucose and fructose yields (92%) achieved following the treatment of nipa sap with 3.0 g/L of

Table 2: Evaluation of Different Catalysts Used for the Hydrolysis of Sucrose in Nipa Sap to Glucose and Fructose and the Resulting pH Values

Catalyst	Hydrolysis Time (min)	Sucrose Conversion (%)	Glucose and Fructose Yield (%)	pH	
				Before Hydrolysis	After Hydrolysis
No catalyst	20	27	25	4.37	4.23
	60	63	61		4.14
	120	86	83		4.04
	180	94	87		3.97
Acetic acid, 3.0 g/L	20	61	61	3.92	3.87
	60	85	82		3.83
	120	98	96		3.80
	180	98	95		3.76
Acetic acid, 10 g/L	20	78	77	3.65	3.62
	60	96	93		3.58
	120	98	93		3.57
	180	99	86		3.56
Oxalic acid, 3.0 g/L	5	92	92	2.67	-
	10	98	98		-
	20	98	98		2.73
	60	98	94		2.73
Oxalic acid, 4.5 g/L	5	98	98	2.10	-
	10	97	96		-
	20	98	94		2.16
	60	98	91		2.17
HCl, 3.0 g/L	5	96	92	-	-
	10	94	87		-
Invertase, 3.92 v/v%	5	73	73	-	-
	15	95	95		-
	30	98	98		-

hydrochloric acid for 10 min were inferior to the sucrose conversion (98%), which indicated that hydrochloric acid could be triggering the decomposition of the monosugars produced during this process. Hydrochloric acid is well known to be highly corrosive [38], and would therefore need to be neutralized or removed from the mixture prior to the acetic acid fermentation process. Based on the disadvantages associated with the use of hydrochloric acid, we concluded that this acid was unsuitable for the hydrolysis of sucrose compared with oxalic acid.

To clarify the kinetics of the acid hydrolysis reaction in the autoclave at 121 °C, it was assumed that the reaction followed first order kinetics as a function of the initial (C_0) and remaining (C_t) sucrose concentrations over time (t):

$$\ln \frac{C_t}{C_0} = -kt \quad (5)$$

Figure 3 shows the effects of various acetic acid and oxalic acid concentrations on the reactions kinetics for the hydrolysis of sucrose. When the hydrolysis was conducted without catalyst (0 g/L), the reaction showed good linearity and therefore appeared to obey first order kinetics. At higher catalyst concentrations, the hydrolysis reactions showed reasonable linearity for the first few min, but not for longer treatment times (e.g., 60 min for 3.0 g/L of acetic acid and 10 min for 3.0 g/L of oxalic acid). The lack of linearity in these cases can be explained in terms of the side reactions occurring under the high temperature conditions, as presented earlier. According to Khajavi *et al.* [39], the hydrolysis reactions of monosugars at high temperatures do not obey first order kinetics because of the acceleration in the rates of the hydrolysis and decomposition reactions with decreasing pH during the hydrolysis. In the case of the hydrolysis of nipa sap without a catalyst, the hydrolysis of sucrose followed first order kinetics, most likely as a result of the relatively slow reaction rate.

Figure 3: Relationship between the C/C_0 ratio of sucrose and the treatment time for the hydrolysis of sucrose in nipa sap by (a) various acetic acid concentrations (0, 3.0, 10 and 20 g/L), and (b) various oxalic acid concentrations (0, 1.5 and 3.0 g/L).

3.3. Enzymatic Hydrolysis

To compare the acid hydrolysis with an enzymatic hydrolysis, we investigated the pretreatment of nipa sap with invertase. As shown in Figure 2c, invertase proved to be an effective catalyst for the hydrolysis of sucrose even at room temperature, with 3.92 v/v% invertase hydrolyzing 98% of the sucrose within 30 min at 25 °C and only 5 min at 60 °C. Both of these temperatures could therefore be used for this process. In addition, the optimum temperature for the fermentation of acetic acid by *M. thermoacetica* is 60 °C [19]. This result therefore indicated that it could be possible to perform the enzymatic hydrolysis together with the fermentation of acetic acid at 60 °C.

3.4. Comparison of the Different Catalysts

Table 2 shows the different catalysts used for the hydrolysis of the sucrose in nipa sap to glucose and fructose, and the resulting pH values. The glucose and fructose yields increased during the initial phase of the hydrolysis, but decreased with prolonged treatment

periods and higher catalyst concentrations. For instance, the glucose and fructose yields for the hydrolysis of nipa sap with 3.0 g/L of oxalic acid decreased from 98 to 94% when the hydrolysis treatment time was extended from 10 to 60 min. Furthermore, increasing the concentration of oxalic acid from 3.0 to 4.5 g/L led to a reduction in the yields of glucose and fructose to 91% after 60 min. For an ideal hydrolysis reaction, the glucose and fructose yields should be equal to the sucrose conversion. However, prolonging the treatment time in the autoclave led to a slight decrease in the total sugar content of the nipa sap. Previous studies involving superheated water have shown that monosaccharides can decompose to give other compounds, such as 5-hydroxymethylfurfural (5-HMF), furfural and organic acids (e.g., formic acid, levulinic acid, acetic acid and lactic acid) [39-41] or isomerize to give different monosugars [42]. According to Nakamura *et al.* [22], organic compounds such as 5-HMF, furfural, lactic acid and formic acid can be fermented by *M. thermoacetica* to acetic acid. Consequently, even if these decomposed products were present in the nipa sap hydrolyzate, they would be fermented to acetic acid by *M. thermoacetica*.

The results presented above for the acid-catalyzed hydrolysis of the sucrose in nipa sap revealed that oxalic acid was more effective than hydrochloric acid because the latter led to the rapid decomposition of the monosugar products. Furthermore, acetic acid was found to be too weak to achieve the rapid and complete hydrolysis of sucrose. It is noteworthy that the pretreatment of nipa sap with oxalic acid has been reported to be less toxic to subsequent biological steps than acetic or sulfuric acids, and does not produce poisonous odors [35]. The optimal conditions for the acid-catalyzed hydrolysis of the sucrose in nipa sap were determined to be 3.0 g/L of oxalic acid within 20 min, which gave 98% yields of glucose and fructose. In the case of invertase, the glucose and fructose yields were equivalent to the sucrose conversion because of the relatively low temperature required for the hydrolysis (25 and 60 °C).

These results revealed that the hydrolytic activities of oxalic acid and invertase were similarly good. Table 3 provides a comparison of oxalic acid and invertase in terms of their price and energy consumption characteristics for the hydrolysis of nipa sap.

Given that the process configurations for the two different types of hydrolysis reaction were different, as shown in Figure 1, the different units of these processes

would have different energy demands. In the case of the acid-catalyzed hydrolysis, the nipa sap was simultaneously sterilized and hydrolyzed in the same unit. In this way, the heat generated during the exothermic hydrolysis of sucrose led to an increase in the temperature of the nipa sap inside the autoclave. Consequently, the autoclave consumed heat energy at a rate of 94.3 MJ/h. However, in the case of the enzymatic hydrolysis, the hydrolysis and sterilization steps were conducted in different units. The hydrolysis step therefore required cooling energy to remove the excess heat formed by the reaction, whereas the sterilization required much greater heat energy than that required for the acid-catalyzed hydrolysis because the heat of the hydrolysis reaction was not recovered. The results presented in Table 3 show that the hydrolysis and sterilization steps consumed energy at rate of -1.9 and 100.6 MJ/h, respectively, for the enzymatic hydrolysis. The negative value in this case shows that heat removal was required.

Table 3: Comparison of Oxalic Acid and Invertase in Terms of their Price and Energy Consumption for the Production of Acetic Acid from Nipa Sap

	Acid Hydrolysis	Enzymatic Hydrolysis
Catalyst	Oxalic Acid Dihydrate	Invertase Solution
Unit price ¹ (\$/kg)	40.0	2,880.0
Flow rate (kg/h)	2.9	31.2
Cost (\$/h)	116.0	89,856.0
Energy Consumption		
Pump (MJ/h)	0.1	0.1
Sterilization (MJ/h)	94.3 ²	100.6
Hydrolysis (MJ/h)	-	-1.9
Total hydrolysis stage (MJ/h)	94.4	100.7
Acetic Acid Fermentation		
Fermentor (MJ/h)	284.3	283.0
Total process (MJ/h)	378.7	383.7

¹These prices were provided by Wako Pure Chemical Industries Ltd. [43,44].

²Energy consumption data for the acid hydrolysis and sterilization (autoclave) steps.

The oxalic acid- and invertase-catalyzed hydrolysis steps consumed 94.4 and 100.7 MJ/h of energy, respectively. Consideration of the whole process including the fermentation step after the acid- and enzyme-catalyzed hydrolysis reactions revealed that their total energy consumptions were 378.7 and 383.7 MJ/h, respectively. There were therefore no major differences

in the energy consumptions of these two hydrolysis methods. However, as shown in Table 3, invertase is more expensive than oxalic acid (approximately 775-fold more expensive). Based on these considerations, the oxalic acid-catalyzed hydrolysis of nipa sap represents the most economically efficient of these two methods. The effects of these two catalysts on the subsequent acetic acid fermentation process will be described in the next section.

3.5. Acetic Acid Fermentation of Hydrolyzed Nipa Sap by *M. thermoacetica*

Nipa sap was hydrolyzed in the presence of 3.0 g/L of oxalic acid at 121 °C for 20 min or 3.92 v/v% invertase at 25 °C for 30 min. The resulting hydrolyzed mixtures were used as substrates for acetic acid with *M. thermoacetica*. Like all known acetogens, the activity of *M. thermoacetica* may be inhibited by high concentrations of acetate ions or protons [45,46]. With this in mind, the total organic concentration of the hydrolyzed nipa sap used in the acetic acid fermentation broth in the current study was decreased to approximately 10 g/L.

Figure 4a shows the batch fermentation profiles for the consumption of glucose and fructose, as well as the production of acetic acid for the nipa sap hydrolyzed by oxalic acid. The results revealed that the substrates had completely fermented after 72 h. However, the acetic acid concentration only increased slightly during the first 24 h, before rapidly increasing to 6.11 g/L over the next 24 h with the highest rate of acetic acid production reaching 0.24 g/L/h. After this point, the production of acetic acid occurred at a much slower rate of 0.02 g/L/h and reached its highest concentration of 10.03 g/L at 217 h. It is noteworthy that the lactic acid and ethanol found in nipa sap may be fermented to acetic acid by *M. thermoacetica* [47], suggesting that these compounds could also be making a small contribution to the final acetic acid concentration.

Furthermore, oxalic acid was not detected in the fermentation broth after 72 h, which indicated that this material was a substrate for *M. thermoacetica*. According to Daniel *et al.* [23], oxalic acid not only supports the growth of *M. thermoacetica* cells in the absence of supplemental CO₂, but is also converted to acetic acid by the bacterium according to the following reaction:

This equation shows that 4 moles of oxalic acid can be converted to 1 mole of acetic acid. A theoretical

Figure 4: Batch fermentation profiles for the production of acetic acid by *M. thermoacetica* from nipa sap following the hydrolysis of the sap with (a) 3 g/L of oxalic acid at 121 °C or (b) 3.92 v/v% invertase at 25 °C.

maximum of 0.04 g/L of acetic acid could therefore be produced from a fermentation broth containing 0.21 g/L of oxalic acid.

The acetic acid yield achieved following the hydrolysis of nipa sap with invertase (Figure 4b) was similar to the yield achieved using oxalic acid as a catalyst for the hydrolysis. The results revealed that all of the glucose and fructose had been completely consumed within 71 h. It is noteworthy, however, that a lag period was observed in the production of acetic acid for the first 23 h, after which time the acetic acid concentration increased considerably to 9.88 g/L over 96 h, with an average acetic acid production rate of 0.10 g/L/h. The maximum acetic acid concentration obtained from 10.22 g/L of organic compounds in the original nipa sap (5.49 g/L sucrose, 2.26 g/L glucose, 2.20 g/L fructose, 0.15 g/L lactic acid and 0.12 g/L ethanol) was 10.05 g/L at 216 h.

Neither of the two catalysts evaluated in the hydrolysis step showed any inhibitory effects towards the subsequent fermentation step with *M. thermoacetica*

and gave similar acetic acid yields of approximately 10 g/L. Research towards the development of a fed-batch fermentation process based on these results is currently underway in our laboratory in an attempt to improve the product concentration.

A comparison of the conversion efficiencies of oxalic acid and invertase for the production of acetic acid revealed that they both gave similarly high values (98%), although the fermentation was slightly faster for the nipa sap hydrolyzed with invertase. It is important to mention that the traditional vinegar production processes used in the Philippines typically produce as little as 4.5–5.5% acetic acid from 15–22% nipa sap because of the CO₂ generated during the fermentation process [8]. These traditional processes are much less efficient than the process developed in this study because they only deliver an average conversion efficiency of 27%. The conversion efficiency of the new process described in this study is 3.6 times greater than that of the traditional methods at 98%. The hydrolytic pretreatment of nipa sap followed by its anaerobic fermentation with *M. thermoacetica* therefore repre-

sents a considerable improvement in the production of acetic acid from nipa sap compared with traditional methods.

The acetic acid generated using our new method could be used directly as vinegar, solvent or preservative in the food industry [15,16,48]. This material could also be used as a key intermediate for the production of other value-added products, such as renewable fine chemicals, pharmaceutical products, plastics, synthetic fibers [16], deicers [49], ethanol fuel via its hydrogenation over a metal catalyst [50-52] and microbial oils for biodiesel production [53].

4. CONCLUSIONS

In this study, we have investigated the hydrolysis and fermentability of nipa sap to give acetic acid. The results revealed that acetic acid acted as an extremely weak catalyst for the hydrolysis step, whereas hydrochloric acid acted as an extremely strong acid catalyst. Oxalic acid and invertase both showed good hydrolytic activities and similar energy requirements for the hydrolysis step, but oxalic acid was determined to be advantageous in terms of its price. Furthermore, oxalic acid may be fermented by *M. thermoacetica*, thereby avoiding the need to separate the catalyst after the fermentation process. The solution generated following the hydrolysis of nipa sap with oxalic acid or invertase was efficiently fermented to acetic acid by *M. thermoacetica*. Compared with traditional methods for the production of vinegar, much higher conversion efficiencies to acetic acid were obtained from nipa sap using the conditions developed in this study. The findings of this study demonstrate that nipa sap and several other sucrose-containing biomass materials can be readily used for the production of acetic acid by acid- or enzyme-catalyzed hydrolysis, followed by fermentation with *M. thermoacetica*, without releasing any CO₂. It is therefore envisaged that this process will not only enhance the economic value of nipa palm in rural coastal communities, but also mitigate environmental burdens associated with this material.

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