Original Paper

Comparative Study of Nutrient Supplements and Natural Inorganic Components in Ethanolic Fermentation of Nipa Sap

Pramila TAMUNAIDU, and Shiro SAKA

(Received October 16, 2012)

The current study was initiated to explore the potential of nipa (*Nypa fruticans*) sap for bioethanol production in the presence of nutrient supplements and natural inorganic components available in the sap. Total chemical composition of nipa sap was 19.5 wt%, mainly composed of sucrose, glucose and fructose. Additionally, the elemental analysis of the sap gave 0.5 wt% inorganics with Na, K and Cl as its main components, corresponding to its habitat. Batch fermentative assays showed that the fermentation trend was similar between nipa and sugarcane sap with over 95% bioethanol yields in conditions with and without nutrient supplements. Further studies showed that, the natural inorganic components available in nipa sap played a significant role as nutrition materials for metabolic growth of microorganisms, consequently affecting the sap fermentability to bioethanol. As a whole, nipa sap showed some interesting characteristics in terms of fermentability without nutrient supplements making it a good alternative feedstock for bioethanol production.

Key Words

Nipa sap, Bioethanol, Inorganic components, Nutrient supplements

1. Introduction

Nipa (*Nypa fruticans*) palm is a vigorously growing palm along river estuaries and mangrove forests with brackish water environment. Nipa palms are known to continuously produce rich sugar-sap from its infructescence for over 50 years.

Generally, collection for sugar source is usually practiced after cutting down the plant, as in sugarcane. However, in the case of nipa, the sugar source in the form of sap, is generally collected from mature infructescence and results in no biomass wastes. Furthermore, the sap collection process does not have any deleterious effect on the palm growth¹⁾. The nipa sap production is recorded at 0.5 to 2.5 L/palm/day with an annual production of 126000 to 169000 L/ha^{1)–4)}.

Nipa palms are distributed all over Asia and northern Australia as natural and plantation-managed palms. The widespread occurrence of nipa palms made rural communities to grow these palms for limited use from sap to make toddy, vinegar or boiled down to sugar with no reported

international trade. Due to its abundance, Food and Agriculture Organization (FAO) has declared this palm as a non-threatened and underutilized palm in South Asia⁵⁾. Therefore, it is our interest to explore the prospect of nipa sap as a potential feedstock for bioethanol production.

Most of bioethanol fermentation processes use nitrogen and carbon compounds as nutrients for efficient and rapid conversions of sugars to ethanol. Nutrient limitation is proposed as a major factor responsible for the decline in fermentative activity during the early stages of fermentation in both *Saccharomyces cerevisiae*⁶⁾ and *Zymomonas mobilis*⁷⁾. However, nutrient supplementation adds fermentation cost and additional cost is necessary to separate these residual impurities after fermentation through membrane technology.

Interestingly, natural sugar feedstocks such as nipa and sugarcane sap should have various nutritive compounds in the form of soluble inorganic components in its sap. While often overlooked as macronutrients, inorganic cations such as Na⁺, K⁺, Ca⁺, Mg²⁺ are required in millimolar concentrations for cellular growth and fulfil specific metabolic and structural roles. Meanwhile micronutrients such as Fe²⁺, Zn²⁺, Mn²⁺ are needed in trace amounts⁸⁾.

Department of Socio-Environmental Energy Science,
Graduate School of Energy Science, Kyoto University
Yoshida-honmachi, Sakyo-ku, Kyoto 606-8501, Japan

Therefore, our study will be on the potential of nipa sap as a feedstock for bioethanol production, mainly focusing on the comparative effect of external nutrient supplements and the role of natural inorganic components available in the sap for its function as nutrition materials. This study will help create the most feasible process for bioethanol production in a rural community. Additionally, development of bioethanol from nipa sap will create awareness in preserving mangrove forests which has become one of the most endangered landscapes and also help alleviate the rampant poverty that plagues the rural community.

2. Material and methods

2.1 Sap collection

The nipa saps used in this study were collected from Pak Phanang Basin, Nakhon Si Thammarat, Thailand. For comparison purposes, sugarcane sap was also studied and obtained from Miyakojima Island, Japan as freshly pressed juice.

2.2 Batch fermentation

Yeast (*Saccharomyces cerevisiae*) (NBRC 0203) (NITE, Japan) was inoculated in Erlenmeyer flasks containing autoclaved medium (glucose 10 g/L, peptone 5 g/L, yeast extract 3 g/L and malt extract 3 g/L, incubated in shaker at 120 rpm for 24 h at 28 °C. Preliminary bioethanol fermentation was conducted by batch using 50 mL fermentation flask. Nutrient supplements of peptone 200 g/L, yeast extract 120 g/L and malt extract 120 g/L was also prepared to assist fermentation or water as non-nutrient treatment to a desired final sugar concentration of 15 wt%. Fermentation was then done in an incubator at 28 °C for a period of 0 to 48 h. Sample of 200 μ L was taken aseptically at appropriate time intervals, filtered using 0.45 μ m Millipore membrane to remove yeast cells for sugar and ethanol analyses.

2.3 Removal of inorganic components

Dialysis was done using seamless, semi-permeable, clear cellulose acetate ester membranes (SpectraPor® Biotech) (Spectrum Labs, USA) with molecular weight cut off (MWCO) of 100 Daltons for the removal of inorganic components in nipa sap. Nipa sap was dialyzed with deionized water for 24 h. Besides dialysis, ion-exchange was done using Supelco Discovery® DSC-NH₂ and DSC-SCX (Sigma-Aldrich, USA). Nipa sap was passed through both these ion-exchangers in series.

2.4 Instrumentation

For individual sugars and ethanol determination in nipa

saps, HPLC coupled with Shodex sugar KS-801 column (Showa Denko, Japan) was used with water as an eluent, running with a flow-rate of 1.0 mL/min under column temperature of 80 °C. Also for organic acids determination, HPLC coupled with Aminex fermentation monitoring column (Bio-rad, USA) was used with 1 mM H₂SO₄ as the eluent, running with a flow-rate of 0.8 mL/min under column temperature of 60 °C. For elemental analysis, a small volume of the sap was incinerated at 600 °C for 2 h and analyzed by scanning electron microscope JSM-5800 (JEOL Ltd., Japan) coupled with energy-dispersive X-ray instrument (Phoenix, USA), at an accelerating voltage of 20 kV. Quantification of inorganic components was done with Shimadzu AA-6300 atomic absorption spectroscopy (AAS) with a highly sensitive graphite furnace atomizer and Agilent G1600A capillary electrophoresis (CE). Additionally, microorganism growth was also analyzed, using visible light at a wavelength of 550 nm on a Shimadzu UV-Vis spectrophotometer 2400 with water as reference.

3. Results and discussion

3.1 Chemical composition

Table 1 shows the chemical composition of nipa sap compared to sugarcane sap. The total chemical composition of nipa sap is of 19.5 wt% mainly composed of sucrose, glucose and fructose. The totals sugars of 18.6 wt% in nipa sap were comparable to 17.0 wt% in sugarcane sap. However, there were no significant difference between these saps in its chemical composition, confining to sugars, organic acids and inorganics.

The ash content described in Table 1 is almost similar in nipa and sugarcane sap at 0.5 and 0.4 wt%, respectively. However to further analyze its individual components, energy-dispersive X-rays (EDX) analyses of the ash obtained from nipa and sugarcane sap were done and its respective spectra is shown as Fig. 1.

The main components in nipa saps were found to be Na, K and Cl, as it grows in swampy saline soils along seashores whereby components like NaCl or KCl naturally

Table 1 Chemical composition of nipa and sugarcane saps

Chemical composition	Yield, wt%	
	Nipa	Sugarcane
Sucrose	11.1	16.4
Glucose	5.9	0.4
Fructose	1.6	0.2
Organic acids	0.4	0.0
Inorganics	0.5	0.4
Total chemical composition	19.5	17.4
Water	80.5	82.6

Fig. 1 EDX spectra of inorganic components obtained as ash from nipa and sugarcane saps

exists in this habitat. Other minor components detected were Ca, Mg, Si, P and S. Thus, inorganic components in nipa sap appear to be affected by its habitation site and its environment acts as natural fertilizers for palm growth. Conversely, major components detected in sugarcane saps were K, Mg, Ca, P and S, corresponding to phosphate and potash fertilizers, calcinated lime, and gypsum used in sugarcane plantations.

3.2 Fermentation of saps with and without nutrient supplementation

Fig. 2 shows the obtained ethanolic fermentation of nipa and sugarcane sap. The dotted line indicates the theoretical maximum amount of ethanol produced after total sugars are fully fermented, which is 0.51 g ethanol/g sugar. This value is used as a basis for calculating the percentage of ethanol conversion from sugars. The ethanol yield

Fig. 2 Ethanol yield for nipa and sugarcane saps as fermented by batch system at 28 °C using *Saccharomyces cerevisiae* with (open symbol) and without (closed symbol) nutrient supplementation

trends were similar for both saps with or without nutrient supplementation.

In nutrient-supplemented media, the fermentation of both nipa and sugarcane sap was rapid in the first 12 h and the fermentation was almost complete after 32 h. The fermentation of nipa sap showed a similar trend to sugarcane sap with 0.494 g ethanol/g sugar, and ethanol conversion of 96.9 %. On the other hand, the obtained ethanol yield from sugarcane sap was 0.495 g ethanol/g sugar.

Interestingly, fermentation could be carried out without nutrient supplementation and both saps showed similar fermentative trends to nutrient-supplemented media. However, the fermentation was apparently slower and ethanol yields of 0.487 and 0.444 g ethanol/ g total sugars were achieved for nipa and sugarcane sap, respectively, after 48 h of fermentation. Without nutrient supplementation, nipa sap is fermented relatively better than sugarcane sap with 95.5 % conversion. Regardless, nipa sap had similar potential for ethanol production as sugarcane sap.

Additionally, nipa and sugarcane saps did not require external nutrients for fermentation and probably utilized naturally available nutrients in the form of inorganic components. However, to further prove this finding, nipa and sugarcane saps without nutrient supplementation were further analysed for individual inorganic components before and after fermentation. The total inorganic components before and after fermentation of nipa and sugarcane sap are shown in Table 2.

As seen in Table 2, the total inorganic components consumed in nipa sap could be noticed with the reduction in its ash content from 4590 to 3804 ppm. High amounts of Na, K and Cl were present in nipa sap corresponding to the EDX spectra as shown earlier in Fig. 1. However, a total of 786 ppm of inorganics was consumed. Minor components of Mg, Ca, P, S and Si present in the saps were in the range between 15 and 192 ppm, and these components were found to be totally consumed or only found in a very small quantity in the sap after 48 h of fermentation. Similarly, inorganic components in sugarcane sap also reduced from 4134 to 2769 ppm after 48 h of fermentation.

Based on this evidence, the presence of inorganic components in nipa and sugarcane sap may have supplemented the growth and metabolism of the microorganism and further assisted in the alcoholic fermentation process. Since our interest is in nipa sap, to further confirm this finding, a study on the effect of inorganic components on the fermentability of the nipa sap was done whereby the sap was treated through dialysis and ion-exchange to remove these components.

Table 2 Inorganic components of nipa and sugarcane saps before and after fermentation without nutrient supplementation

	Inorganics, ppm	Inorganic components in sap, ppm							
		Na	K	Mg	Ca	P	S	Si	Cl
Nipa									
Before	4590	1212	1780	77	15	140	192	65	1109
After	3804	1119	1699	7	n.d.	n.d.	5	n.d.	974
Sugarcane									
Before	4134	120	2200	279	750	270	355	50	110
After	2769	20	2159	20	450	n.d.	70	n.d.	50

n.d.; not detected

3.3 Inorganic components removal through dialysis and ion-exchange

Membrane dialysis and ion-exchange are widely used in scientific research in the removal of inorganic components. The effect of these treatments on the inorganic components content present in nipa saps is tabulated in **Table 3**. The original sap had 4590 ppm of inorganics and their detailed components are shown to compare the significant reduction of their content through the specified treatments. Almost all inorganic components are removed through these treatments with ion-exchange removing 93 % of inorganics from the sap, leaving a trace of only 340 ppm of inorganics.

On the other hand, membrane dialysis removed only 55 % of the total inorganics with 2080 ppm of inorganics still remaining after 24 h dialysis with water. The major content of inorganics remained after dialyses were 1068 ppm of Na and 1010 ppm of K. These components were also found after ion-exchange with lower quantity of 100 ppm of Na and 240 ppm of K. However, collectively, these treatments removed most of the minor components like Mg, Ca, P, S and Si as well as one of the major element, Cl.

3.3.1 Fermentation of treated nipa saps

All nipa saps; original and treated (dialyzed and ion-exchanged) saps, were subjected to batch ethanol fermentation. Any sugar loss due to dialysis and ion-exchange is compensated with sucrose and the initial sugar concentration of all saps is adjusted to 15 wt% before fermentation. **Fig. 3** shows the obtained ethanol yield of nipa saps upon total sugars as fermented at 28 °C using yeast (*Saccharomyces cerevisiae*) without nutrient supplementation. Pure sucrose

fermentation was done for comparison, in which no inorganic components exist.

As observed in **Fig. 3**, the ethanol yield trends were similar for all saps without nutrient supplementation. However, the ethanol yields were lower in the treated saps compared to the original nipa sap. The obtained ethanol yield from original nipa sap was 0.487 g ethanol/g sugar, with ethanol conversion from 95.5 % at 48 h. Conversely, only 0.266 g ethanol/g sugar was obtained in dialyzed sap and 0.092 g ethanol/g sugar in ion-exchanged sap.

The ethanol yields reduced significantly corresponding to the inorganics content removed through dialysis and ion-exchange. Similarly, the fermentation of pure sucrose without nutrients was too slow and only 0.011 g ethanol/g

Fig. 3 Ethanol yield for nipa saps after removal of inorganic components through dialysis and ion-exchange as fermented without nutrient supplementation. Original sap and sucrose fermentation were made for comparison

Table 3 Inorganic components of original nipa sap compared to dialyzed and ion-exchanged nipa sap

	Inorganics, ppm	Inorganic components in sap, ppm							
		Na	K	Mg	Ca	P	S	Si	Cl
Original	4590	1212	1780	77	15	140	192	65	1109
Dialyzed with water for 24 h	2080	1068	1010	n.d.	n.d.	n.d.	n.d.	2	n.d.
Ion-exchanged	340	100	240	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

n.d.; not detected

sugar was achieved at the end of 48 h.

An observation by Akrida-Demertzi and Koutinas showed that the fermentation rate increased with the increase in inorganic components from 0 to 5 ppm Cu, 1000 ppm Ca, 5 ppm K, and 5 ppm Na⁹⁾. These concentrations of Cu, Ca, K, and Na were optimum for the alcoholic fermentations of sucrose solutions. Dombek and Ingram also have observed that Ca and Mg have protective effects on microorganism growth which stimulate the fermentation rate and ethanol production¹⁰⁾.

As clearly shown in Fig. 3, the original nipa sap with the highest inorganics content produced the highest ethanol yield. It did not require external nutrients for fermentation and utilized naturally available nutrients in the form of inorganic components as also shown in Table 2. Based on these lines of evidence, the presence of these components in the sap and the reduction in its content observed after fermentation proves that the natural inorganic components assisted the alcoholic fermentation process.

3.3.2 Microorganism growth

To further confirm the role of inorganics on the growth of the microorganism, all saps were analyzed at each time period with the increase in ethanol concentration. Profiles of microorganism growth were plotted against ethanol concentration for all saps as shown in Fig. 4. Here, the microorganism growth is expressed as absorbance at 550 nm.

For each sap with increasing inorganics content, the ethanol yield increased with the increase in microorganism growth. Minimal growth was observed in ion-exchanged sap with 340 ppm of inorganics content, while the largest growth observed in the original sap with 4590 ppm of inorganics content.

The maximum microorganism growth in original and

treated nipa saps directly corresponds to the highest ethanol yield achieved in its respective sap. However, the decrease in ethanol yields in dialyzed and ion-exchanged saps are largely due to limited nutritional resources for metabolic growth of microorganisms. Consequently, the growth rate of microorganisms was affected and they performed poorly resulting in reduction in ethanol yields. These findings indicate that nutrient deficiency is an important factor in decreasing the rate of fermentation.

However, this nutrient deficiency is from the reduction in natural inorganic components available in nipa sap through dialysis and ion-exchange. Therefore, the natural inorganic components in nipa sap are proven to supplement the growth of microorganisms and consequently increase bioethanol productions. This comparative study showed that the natural inorganic components in nipa sap are equivalent to nutrient supplements provided for fermentation of sugar feedstocks.

4. Conclusions

Nipa sap was rich in sucrose, glucose and fructose further found to be fermented to ethanol in high yields in conditions with and without nutrient supplementation. Natural inorganic components available in nipa saps enhanced the ethanolic fermentation without nutrient supplementation. As nutrients are generally required in the fermentation process, alcoholic fermentation without external nutrients is, therefore, economical for ethanol production with nipa saps. Even though additional hours are needed for complete fermentation without nutrients, the fermentation time is still reasonable. Additionally, the cost of removal of residual impurities from nutrients after the fermentation can also be avoided. These characteristics of nipa sap make it a good alternative feedstock for development of bioethanol production in rural coastal communities.

Acknowledgements

We would like to thank Dr. Hitoshi Miyasaka and Mr. Keiyo Morimune from Environmental Research Center, The Kansai Electric Power Co. Inc, Kyoto, as well as Dr. Naohiro Matsui and Dr. Yasuyuki Okimori from The General Environmental Technos Co. Ltd, Osaka, for their generous contribution towards this research.

References

- 1) Dennett, J., *Malay. Agric. J.*, **15**, 420-43 (1927)
- 2) World Agroforestry Centre, Available online at <http://www.worldagroforestrycentre.org/sea/Products/AFDbases/AF/asp/SpeciesInfo.asp?SpID=18140>, (2008), [Last accessed: Dec. 24, 2012]

Fig. 4 Profile of microorganism growth at various ethanol yields for original, dialyzed and ion-exchanged nipa saps. Absorbance was made at 550 nm.

- 3) Päivöke, A. E. A., *Agric., Ecosyst. Environ.*, **13**, 59-72 (1985)
 - 4) Gibbs, H., *Philipp. J. Sci., Sect. A*, **6**, 99-206 (1911)
 - 5) Food and Agriculture Organization of the United Nations, Available online at <http://www.fao.org/docrep/X0451E/x0451e00.HTM>, (1998), [Last accessed: Dec. 24, 2012]
 - 6) Dombek, K. M., Ingram, L. O., *J. Ind. Microbiol.*, **1**, 219-225 (1986)
 - 7) Doelle, M. B., Doelle, H. W., *J. Biotechnol.*, **11**, 25-36 (1989)
 - 8) Robin Robinett, R. S., George, H. A., Herber, W. K., *J. Chromatogr., A*, **718**, 319-327 (1995)
 - 9) Akrida-Demertzi, K., Koutinas, A. A., *Dev. Food Sci.*, **29**, 475-489 (1992)
 - 10) Dombek, K. M., Ingram, L. O., *Appl. Environ. Microbiol.*, **52**, 975-981 (1986)
-