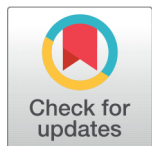


## RESEARCH ARTICLE



### OPEN ACCESS

Received: 27-07-2020

Accepted: 09-08-2020

Published: 31-08-2020

Editor: Dr. Natarajan Gajendran

**Citation:** James Paul T. Madigal, Jayson F. Cariaga, Thiara Celine E. Suarez, Shirley C. Agrupis (2020) Assessment of the Physico-chemico and Microbiological changes during Nipa (*Nypa fruticans*) Sap Fermentation collected from different sites in Cagayan province, Philippines. Indian Journal of Science and Technology 13(32): 3230-3236. <https://doi.org/10.17485/IJST/v13i32.1251>

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**Funding:** National Bioenergy Research and Innovation Center

**Competing Interests:** None

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Published By Indian Society for Education and Environment ([iSee](#))

ISSN

Print: 0974-6846

Electronic: 0974-5645

## Assessment of the Physico-chemico and Microbiological changes during Nipa (*Nypa fruticans*) Sap Fermentation collected from different sites in Cagayan province, Philippines

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### Abstract

**Background/Objectives:** Sap from *Nypa fruticans* are used as feedstock for a bioethanol national program to establish a sustainable and productive village-scale bioethanol industry. The study aimed to establish the physico-chemical, microbiological, and molecular characteristics of nipa sap. **Methods/Statistical Analysis:** Nipa sap samples were collected from three sampling sites at different fermentation time. Physico-chemical parameters such as pH, titratable acidity, and total soluble solids were determined and statistically analyzed using One-way Analysis of Variance and mean  $\pm$  SD values. Molecular approaches were used to profile and identify yeast species naturally residing in the sap during fermentation and assess the genetic diversity of nipa palm in selected sampling locations. **Findings:** Sampling site and fermentation time significantly affect the levels of pH, titratable acids, and total soluble solids in the sap samples. Sugar concentration in nipa sap is affected by sampling site: as the sampling site nears the river, the amount of sugars become significantly greater. Molecular studies revealed that among the 12 isolates obtained, five of them (Y24S1, Y48S1, Y72S1, Y24S2, and Y24S3) showed 100% similarity to strain of *Pichia occidentalis*, three (Y48S2, Y48S3, and Y72S3) were similar (100%) to a strain of *Saccharomyces cerevisiae* (YPDW9), and the other two isolates (Y72S1 and Y48S3A) were identical to *Naganishia randhawae* strain HBUMO6885 and *Tremellales* sp. LM523 respectively with a 100% similarity. PCR amplification of their trnL-trnF cpDNA genome, BLAST and phylogenetic analyses reveals that all nipa plant samples were similar to *Nypa fruticans*. **Applications:** The generated information on the effect of sampling site on sugar content of nipa sap samples could be good input in selecting expansion sites for the establishment of nipa plantations. Furthermore, the identified yeast strains can be used as inoculant or starter culture to improve fermentation.

**Keywords:** Physico-chemical parameter; yeasts molecular profiling; nipa sap fermentation; phylogenetic analysis; genetic diversity

## 1 Introduction

Nipa palm (*Nypa fruticans*) is the most promising of first-generation feedstocks owing to its advantages over other fuel crops. First, it does not compete with food crops for land and water resources because it thrives where most crops cannot grow; second, it requires very little maintenance because once established, it will last for at least 50 years - in contrast with all other sources of bioethanol that need to be replanted after harvest; third, it has many other uses in such innovative systems as aquasilviculture designed to rehabilitate abandoned fishponds where fishery and nipa production complement each other<sup>(1,2)</sup>.

*Nypa fruticans* is known to continuously produce rich sugar-sap from its infructescence for over 50 years. The sap is collected from mature infructescence and does not have any harmful effect on the palm growth. The estimated annual ethanol yield of 3,600–22,400 L<sup>-1</sup> ha<sup>-1</sup> year<sup>-1</sup> from nipa sap makes it an attractive raw material for ethanol production compared to sugarcane and corn, with reported yields of 5,300–6,500 and 3,100–3,900 L<sup>-1</sup> ha<sup>-1</sup> year<sup>-1</sup>, respectively<sup>(3)</sup>. Fermented nipa sap has complex organic and inorganic compounds; the production of which depends on many factors, including period of fermentation and microbial activity. In general, fermented palm sap contains low molecular mass carbonyls (C<sub>1</sub> -C<sub>6</sub>) as by-products of yeast fermentation, and alcoholic oxidation at various stages of fermentation<sup>(4)</sup>.

Several efforts have been carried out worldwide to improve the efficiency of bioethanol production, e.g. substrate optimization, process improvement, and performance enhancement of microorganisms during fermentation<sup>(5,6)</sup>. The present study was conceptualized to generate fundamental knowledge on the characteristics of nipa sap, fermenting microorganisms, and the nipa plant itself that can affect the quantity of sugar and bioethanol.

The present study generally aimed to establish the physico-chemical and molecular characteristics of nipa sap which could be used as bases for the development of a more focus R&D roadmap for much improved nipa sap productivity and optimum bioethanol production. Specifically, the study aimed to: determine and compare the physico-chemical characteristics of the fresh and fermented nipa sap as affected by fermentation time and location; assess the genetic diversity of nipa from the various sampling locations; establish the relationship between the genetic variability and the nipa sap sugar; and isolate, characterize, and compare the yeasts from nipa sap fermentation collected from different sampling site using their Internal Transcribed Spacer (ITS) region.

## 2 Materials and Methods

### 2.1 Collection and preparation of Nipa sap samples

The nipa sap samples were collected from Barangay Cabaggan, Pamplona, Cagayan Valley, Philippines in three pre-identified sampling sites with respect to their proximity to bodies of water. Site 1 is located in the riverbank 2 m away from the river and is always submerged in the water. Site 2 was established 50 m. away from the first site where the plants are not submerged in water. Site 3 was located 50 m. away from Site 1 and 2 and is only flooded with water when the water level in the river rises.

Sap samples were collected following the MMSU USAID-STRIDE Nipa Projects' traditional protocol for preparing the peduncle prior to sap collection. Collected samples were subjected to physic-chemical and microbiological analyses. Potassium metabisulfite was added to samples for fresh sap analysis in order to arrest the fermentation. Another set of containers was utilized to collect sap samples that will undergo fermentation prior to analysis at three fermentation periods, i.e., 24, 48 and 72 hours. The samples for the 0-hour fermentation period were collected using sterile bottles and stored in an ice chest to arrest fermentation. Sampling was done in three replicates.

### 2.2 Analysis for the physico-chemical properties of Nipa sap

Quantitative research protocols were used to determine the physico-chemical properties of the nipa sap (fresh and fermented sap) as affected by time and location. The pH values were measured at ambient temperature with a pH-meter. Titration was used to determine the total acidity of all samples using 0.067 N NaOH titrant solution. The volume of the titrant used was recorded and the amount of titratable acids (TA) in g/L tartaric acid was equal to the volume of NaOH used during the titration. The fermented samples were degassed prior to titration to minimize carbon dioxide interference by heating the samples to almost boiling state, then agitated and were allowed to cool down.

The total soluble solids of nipa sap were determined using hand refractometer. Total sugars found in the sap was determined using the Dinitrosalicylic Acid (DNS) analysis. The calibration curve was constructed using glucose as a standard. Different dilutions were done in different concentrations ranging from 0 (blank), 0.2, 0.4, 0.6, 0.8 and 1.0 from a stock glucose solution (1 mg/ml). Absorbance was read at 540 nm wavelength. Then, 1 ml of each of the sap samples were placed in separate test tubes. After this, 2.5 ml of DNSA reagent were added to each test tube and were placed in a boiling water bath for 15 minutes. The samples were allowed cooled down to room temperature prior to reading the absorbance at 540 nm. All physico-chemical parameters data were analyzed using One-way Analysis of Variance and mean  $\pm$  SD values.

### 2.3 Microbiological analysis of Nipa sap

Analysis of the microbial composition of the nipa sap fermentation focuses only on the yeasts profile of the nipa sap. Molecular identification was used to determine the strain of yeasts from nipa sap fermentation collected from the three sites in Barangay. Cabaggan, Pamplona, Cagayan, Philippines.

Samples were diluted up to 10<sup>-4</sup> using 0.85% NaCl solution. One microliter was spread onto Yeast Extract Peptone Dextrose Agar (10 g L<sup>-1</sup> yeast extract, 20 g L<sup>-1</sup> peptone, 20 g L<sup>-1</sup> dextrose, and 15 g L<sup>-1</sup> agar supplemented with 0.003% Rose Bengal and 100 ppm streptomycin)

plates. The plates were incubated for 48 hours at 30°C<sup>(7)</sup>. The colonies that showed different cultural characteristics like form, color, elevation, margin, texture and surface were observed and selected for the purification method.

Cultures that exhibited different morphological features were sent to First BASE Laboratories Sdn Bhd located at Taman Serdang Perdana, Seksyen 2, 43300 Seri Kembangan, Selangor, Malaysia, and to subjected to DNA extraction and molecular identification by sequencing. The generated sequences were compared to the existing database (GenBank) using the Basic Local Alignment Search Tool (BLAST). Identity that was given by BLAST results were considered significant if the difference between the unknown isolate and match is 1% or less<sup>(8,9)</sup>. The result with the highest maximum score per isolate was also considered.

## 2.4 Genetic diversity analysis of Nipa plant samples

Nipa leaves were collected from three different sites in Barangay Cabaggan, Pamplona Cagayan. Three leaf samples were obtained in each sampling site and keep a sterile zip lock. Samples were stored in an ice chest and sent to the Philippine Genome Center (PGC), UP Diliman, Quezon City for genetic diversity analysis of the nipa plant which include, DNA extraction, PCR amplification, and sequencing analysis. phylogenetic analysis was done using MEGA7 (Molecular Evolutionary Genetic Analysis)<sup>(8,9)</sup>. The sequences were aligned using Clustal W and a phylogenetic tree was constructed using the Neighbor-joining method. The associated taxa are clustered together using bootstrap test at 1000 replications.

## 3 Results and Discussion

### 3.1 Changes of the physico- chemical properties of Nipa sap during fermentation

#### 3.1.1 Changes in pH levels of the Nipa sap overtime

The physico-chemical properties of sap samples were measured at different fermentation times namely: 0, 24, 48 and 72 hours. The pH levels of the samples collected from each sampling site were measured using a portable pH meter. Results revealed that sampling site and fermentation time significantly affect the pH levels of the sap samples, but there is no significant difference between the pH levels of the sap and the combined interaction of the effects of sampling site location and time ( Table 1). Nipa sap collected in a place where the nipa plant is always submerged in water have a significant lower pH mean values, which is significantly more acidic than the pH of the samples collected from the two other sites ( Table 1).

As the natural fermentation process of the sap occurs, ethanol, which is a weak acid is gradually produced. As seen in Table 1, it can be deduced that nipa plants that are closer to the river produce ethanol in significantly greater amounts than in locations located farther away from the body of water because the lower the pH value, the more acidic the sample is and therefore, there is more ethanol in it. On the other hand, for the effect of fermentation time on the pH of the sap samples, significant differences in the pH levels of the samples were observed only after 72 hours (4 days). Data suggests that the distillation of nipa sap should only then happen 4 days after the collection of the sap to maximize ethanol yield.

#### 3.1.2 Total acidity of the Nipa sap overtime

Total acidity (TA) was measured in g/L tartaric acid using titration utilizing a 0.067M NaOH solution slowly added to the samples until pH levels reached beyond pH 8.2. Data showed that the sampling site and fermentation time significantly affect the TA of the sap samples, but there is no significant difference between the TA of the sap and the combined interaction of the effects of sampling site location and time ( Table 1).

Table 1. Changes in physico-chemical parameters of sap samples in different sampling sites under aerobic condition overtime

Factors	pH	TA	TSS
SITE	**	**	**
1	4.51 <sup>b</sup>	4.33 <sup>a</sup>	4.33 <sup>b</sup>
2	4.84 <sup>a</sup>	3.32 <sup>b</sup>	4.33 <sup>b</sup>
3	4.78 <sup>a</sup>	4.58 <sup>a</sup>	5.08 <sup>a</sup>
TIME	**	**	**
0hr	4.82 <sup>a</sup>	5.77 <sup>a</sup>	5.11 <sup>a</sup>
24hr	4.94 <sup>a</sup>	2.73 <sup>c</sup>	4.56 <sup>b</sup>
48hr	4.78 <sup>a</sup>	3.63 <sup>b</sup>	4.22 <sup>b</sup>
72hr	4.31 <sup>b</sup>	4.17 <sup>b</sup>	4.44 <sup>b</sup>
SITE:TIME	ns	ns	ns
CV, %	4.06	17.56	8.91

CV: coefficient of variation

\*\*significant at P<0.01, values with the same letter are not significantly different

Noticeably, the sampling site affects the total acidity of the sap samples. Samples collected from Site 2, which are not submerged in water, are significantly less acidic, with mean TA of 3.32, than the samples from the other two sites (Table 1). pH and titratable acids are two entities

which measure separate parameters. pH is the logarithmic measure of the concentration of free hydrogen ions in a chemical or biological system. On the other hand, TA is a simple measure of the acid anions in a juice. Hence, pH and titratable acidity do not measure the same thing. Although samples from Site 1 have the highest pH values because they are submerged in water, samples collected from the second site have significantly more titratable acids in them because they are in a relatively drier environment as they are not submerged in water.

For the change in the titratable acids over time, the TA values observed to be higher during the initial hours upon sap collection and decreased over time. The decrease in titratable acidity is caused by the precipitation of potassium bitartrate, which becomes less soluble with increasing ethanol concentration as fermentation proceeds over time. When it precipitates, it removes a proton from the solution that would otherwise have contributed to the TA concentration, thereby decreasing the TA over time.

### 3.1.3 Total soluble solids of the Nipa sap overtime

A hand refractometer was used to measure the Total Soluble Solids (TSS) of the collected nipa sap. Sugar concentrations are expressed in degrees Brix at 20°C where Brix is usually considered equal to the percentage of sugar in the solution. The sampling site and fermentation time significantly affect the TSS of the sap samples, but there is no significant difference between the TSS of the sap and the combined interaction of the effects of sampling site location and time. Site 3 has a significantly higher TSS mean value (5.08°/100ml) than the samples in both Sites 1 and 2 with the same mean value of 4.33°/100ml (Table 1).

For the different fermentation time regimes, the total soluble solids significantly decreased over time. This is because as the process of fermentation proceeds, the sugars present in the sap are being degraded into ethanol and carbon dioxide, therefore, the amount of sugars in the sap are decreasing. The results show that the sampling site affects the total sugar content of the nipa sap. However, since the refractometer not only measures the amount of sucrose in the samples but also detects all the soluble solids present in the sample that have the ability to refract light, then a more accurate method of detecting sugars in the samples should also be done.

## 3.2 Effect of sampling location on the sugar concentration of Nipa sap

Quantification of the amount of reducing sugars present in sap samples collected from different sampling sites was conducted using Dinitrosalicylic Acid (DNS) method. Sugar content of nipa sap (3.25mg/100ml) collected from Site 1, nipa plants that are always submerged in water and two meters away from the river, is significantly higher than those from Site 2 (0.62mg/100ml) and Site 3 (1.11mg/100ml). Nipa plants in Site 2 are not submerged in water while in Site 3, they are only flooded with water occasionally when the water level in the river rises (Table 2). Hence, sugar concentration is a function of the nipa plantation site. When the plant is located more proximal to the river, the sugar concentrations in the sap are relatively higher.

Table 2. Reducing sugar concentrations of the sap samples collected from different sampling sites.

Sampling site	Concentration (mg/100ml)
1	3.25 a
2	0.62 b
3	1.11b

Significance:\*\*; significant at P<0.01, ns; not significant

Physico-chemical parameters in nipa sap samples are affected by the location of the site with respect to its proximity to bodies of water, and by different fermentation time regimes. Fluctuations in the values are caused by the processes occurring after the sap is collected and is therefore exposed to air, allowing fermentation to proceed, and by the proximity of the plant to water sources.

## 3.3 Molecular characterization and identification of Nipa Yeast isolates

### 3.3.1 Internal transcribed spacer (ITS region of the Nipa Yeast isolates)

A total of 12 yeast isolates with different colony morphologies were obtained and purified. These 12 yeast isolates were subjected to DNA extraction and internal transcribed spacer-polymerase chain reaction (ITS-PCR) to determine their identities.

ITS region amplified by PCR yielded seven isolates with same length of amplicons and three yielded different length of amplicons. Seven yeast isolates at different fermentation times coded as: 24h (Y24S1, Y24S2, and Y24S3); 48h (Y48S1, Y48S3, and Y48S4); and 72h (Y72S2) yielded an amplicon size of ~500 bp, while the three yeast isolates coded as: 48h (Y48S2) and 72h (Y72S1 and Y72S3) showed different amplicon length of ~850 bp.

ITS-PCR allows for differentiation of species within a genus but not strains within the same species because of its high interspecific variability<sup>(10)</sup>. When resolved in ITS gel, lengths of the ITS regions are said to be homologous, with the same size. Therefore, it is highly possible that the seven representative isolates (Y24S1, Y24S2, Y24S3, Y48S1, Y48S3, Y48S4 and Y72S2) are of the same species and the other three isolates (Y48S2, Y72S1 and Y72S3) belong to different species. Sequencing analysis of the ITS amplicons was done to further verify the identities of the isolates.

### 3.3.2 BLAST analysis of ITS profile

The 5.8S-ITS sequences of the isolates were subjected to BLAST to determine their identities. Table 3 shows the strains which shared the closest identity to the 12 isolates profile from their 5.8S-ITS sequences.

Noticeably, five isolates (Y24S1, Y48S1, Y72S1, Y24S2, and Y24S3) showed 100% similarity to *Pichia occidentalis* isolate KOP7. Three isolates (Y48S2, Y48S3, and Y72S3) showed their similarity (100%) to a strain of *Saccharomyces cerevisiae* (YPDW9). Other two isolates (Y72S1 and Y48S3A) were identical to two strains of yeasts (*Naganishia randhawae* strain HBUMO6885 and *Tremellales* sp. LM523) with 100% similarity (Table 3).

The data indicate the transition and diversity of yeasts strains in all fermentation time. Based on the BLAST analysis, strain of *P. occidentalis* can be observed from 24hr fermentation time onwards, while strain of *Saccharomyces cerevisiae* appeared from 48 hr to 72 hr fermentation. Two strains of yeast were observed in the later fermentation time: *Naganishia randhawae* strain HBUMO6885 and *Tremellales* sp. LM523. *N. randhawae* was isolated and observed in fermented grains while the *Tremellales* sp. was from inland sea water. A recent study revealed four strains of yeasts from fermenting nipa sap collected from two selected sites in the Philippines. Molecular identification showed three strains of *S. cerevisiae* and one *P. kudriavzevii* strain<sup>(11)</sup>.

**Table 3.** Possible identities of 12 isolates after 5.8S-ITS rDNA sequencing and BLAST analysis

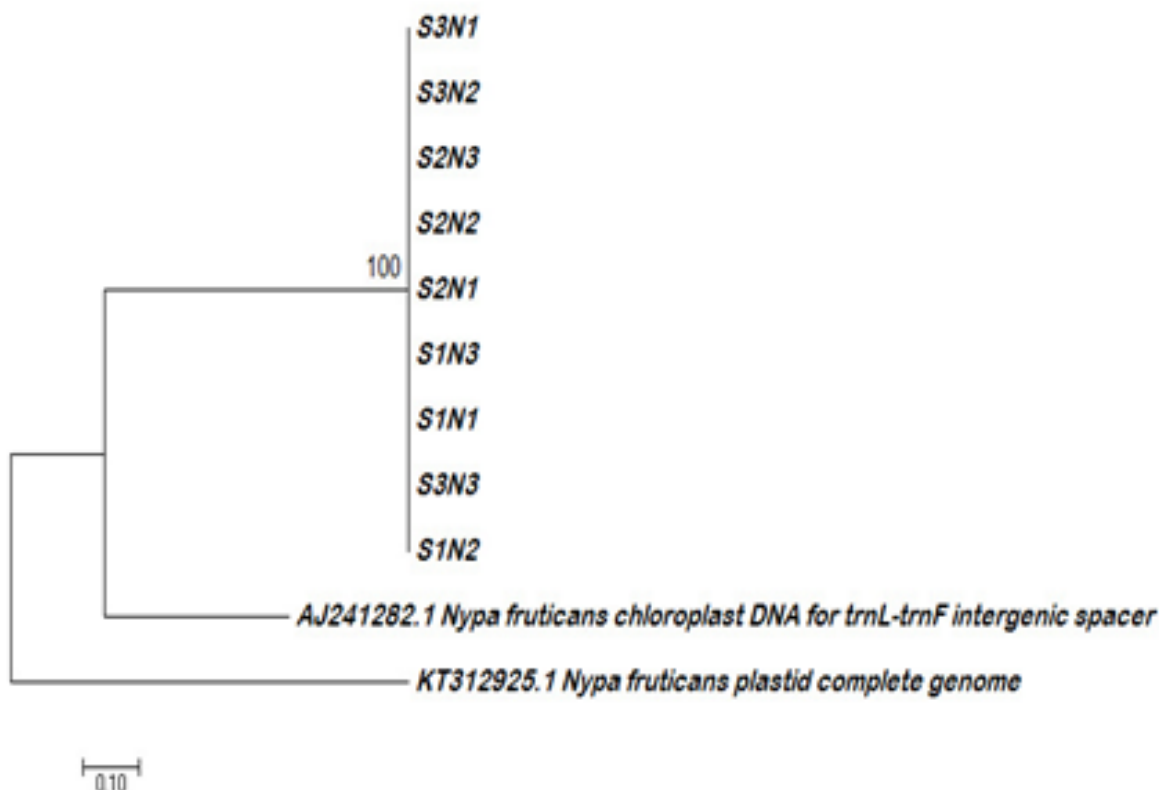
Isolate Code	Sampling Site/ Fermentation time (h)	Yeast Identified (Closest Match using BLAST)	% Similarity	Accession number
Y24S1 and Y48S1	Site 1 24, 48, and 72	<i>Pichia occidentalis</i> isolate KOP7	100	KY849376.1
Y72S1	Site 1 72h	<i>Naganishia randhawae</i> strain HBUMO6885	100	MF662353.1
Y24S2	Site 2 24	<i>Pichia occidentalis</i> isolate KOP7	100	KY849376.1
Y48S2	Site 2 48	<i>Saccharomyces cerevisiae</i> strain YPDW9	100	KY816904.1
Y24S3	Site 3 24	<i>Pichia occidentalis</i> isolate KOP7	100	KY849376.1
Y48S3, and Y72S3	Site 3 48 and 72	<i>Saccharomyces cerevisiae</i> strain YPDW9	100	KY816904.1
Y48S3A	Site 3 48	<i>Tremellales</i> sp. LM523	99	EF060814.1

Hence, the data suggest a confirmation study on the yeasts profiling of nipa sap fermentation at different sampling site using Denaturing Gradient Gel Electrophoresis (DGGE) to validate the transition of yeast profile in different fermentation time and sampling site.

### 3.4 Effect of Nipa plant genetic diversity in the sugar content of Nipa sap

The nipa leaf samples collected from different sampling sites were sent to PGC for DNA extraction and further analyses such PCR amplification and sequencing. Sequencing and phylogenetic analyses reveals that all samples obtained from the three different sampling sites are similar to *Nypa fruticans* with 100% bootstrap. Hence, all nipa plants in the sampling sites of the study are populated by only one species of nipa palm.

Furthermore, differences in the sugar content of nipa sap from three collection sites are not influenced by genetic diversity. It is emphasized that such analysis prelude conducting similar studies in other nipa populations.



**Fig 1.** Dendrogram showing the phylogenetic relationship among the unknown Nipa plant DNA sequences (S1N1, S1N2, S1N3, S2N1, S2N2, S2N3, S3N1, S3N2, and S3N3) with the two known species of nipa plant (*Nypa fruticans* chloroplast DNA and *Nypa fruticans* plastid complete genome). The tree was constructed using Maximum-Likelihood-Joining Method in MEGA7 Bootstrap values, expressed in percentages, are shown next to the branches.

Legend: S1N1, S1N2, and S1N3 = Nipa leaf samples from Site 1 in triplicate  
 S2N1, S2N2, and S2N3 = Nipa leaf samples from Site 2 in triplicate  
 S3N1, S3N2, and S3N3 = Nipa leaf samples from Site 3 in triplicates

## 4 Conclusion

Results revealed that chemical characteristics of nipa sap samples are affected by the location of the site and by different fermentation time. Fluctuations in the values are also caused by the processes occurring after the sap is collected, including exposure to air and time allowed for fermentation to proceed. Sugar concentration is a function of the site. Nipa palms nearer the river (Site 1) produced more sugar in the sap than those further away (Sites 2 and 3). Genetic diversity analysis showed that the collected nipa plant samples belong to *Nypa fruticans*. Hence, genetic diversity is not a contributory factor to the differences in the sugar content of sap samples taken from the three collection sites. Two yeast strains were isolated from the collected Nipa sap. Through ITS, these were identified as *Pichia occidentalis* and *Saccharomyces cerevisiae*. The former was dominant in all of the sampling sites at different fermentation time while the latter was isolated from Sites 2 and 3 only.

The generated information on the effect of sampling site on sugar content of Nipa sap samples could be good input in selecting expansion sites for the establishment of Nipa plantations. On the other hand, the identified yeast strains can be used as inoculant or starter culture to improve fermentation.

## Acknowledgement

The authors would like to thank National Bioenergy Research and Innovation Center for funding and hosting the conduct of this research. Also, the authors would like to thank Philippine Genome Center, UP Diliman and First Base Molecular Biology and Laboratory Services, Malaysia for the molecular biotechnology analysis.

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