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Isolation and Evaluation of Stress-Tolerant Indigenous Yeast Isolates from Naturally Fermenting Nipa (Nypa fruticans Wurmb.) Sap for Potential Bioethanol Production

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ABSTRACT: This study aimed to isolate and characterize stress-tolerant yeasts from nipa (Nypa fruticans Wurmb.) sap for bioethanol production, addressing the Philippine bioethanol industry's need for alternative feedstocks and stress-tolerant yeast strains. Two dominant putative yeast strains (NY1 and NY2) were isolated from 72-hour naturally fermenting sap collected in Calizo, Balete, Aklan. These indigenous isolates were evaluated for thermotolerance, osmotolerance, ethanol tolerance, and acid tolerance, and compared against a commercial Brewer's yeast (Saccharomyces cerevisiae) control. The indigenous isolates demonstrated greater early-stage stress resistance, exhibiting higher osmotolerance (up to 20% sucrose) and acid tolerance (thriving at pH 2.0–4.0) than the commercial strain. Although the commercial control showed greater overall resilience to late-stage ethanol concentrations and heat (45°C), isolate NY1 exhibited adaptive thermotolerance at 40°C. Laboratory-scale batch fermentation assays (125 mL working volume) indicated the ethanologenic potential of both isolates. In a Simulated Nipa Sap (SNS) medium, NY1 and NY2 achieved mean ethanol yields of 3.66% v/v (4.57 mL) and 3.28% v/v (4.10 mL), respectively, which were higher than their observed yields in a standard YPD medium (3.36% and 3.04% v/v). Ethanol production was verified via Fourier-Transform Infrared (FTIR) spectroscopy. These preliminary findings suggest the potential of indigenous nipa sap yeasts for bioethanol applications. Future research should prioritize the molecular identification of these isolates and the scaling up of fermentation processes to evaluate their suitability for industrial application.

1. INTRODUCTION

Bioethanol is an alcohol made by microbial fermentation, mostly from carbohydrates produced in sugar- or starch-bearing plants like corn, sugarcane, sweet sorghum, or lignocellulosic biomass (Anyanwu et al., 2022). It is one of the most promising alternatives among sustainable biofuels (Kaymak, 2017), known to significantly reduce greenhouse gas emissions while improving vehicle engine efficiency (Zaldarriaga, 2026).

The enactment of Philippines RA 9367, also known as the Biofuels Act of 2006, makes the country the pioneer in biofuels blending in Southeast Asia. The law which mandates a 10% bioethanol blend in gasoline (Gatdula, Demafelis, and Battaler, 2021). To further mitigate high fossil fuel costs, the Department of Energy recently approved the roll-out of a voluntary 20% blend, or E20 (USDA, 2024). With global supply chain issues causing unstable imported oil prices, a strong local biofuel industry acts as a necessary economic buffer. Increasing local production helps control fuel-driven inflation, guarantees steady market demand for local farmers, and secures the country's long-term energy supply (Zaldarriaga, 2026).

However, the two major gaps in the Philippine bioethanol industry are the insufficient feedstock availability and the high domestic ethanol price due to the inefficiency of the process (Gatdula, Demafelis and Bataller, 2021). The main feedstocks for bioethanol are sugarcane-based materials, which are molasses and sugarcane juice. While 80% of total molasses in the country is used for ethanol production, limited feedstock continues to be a challenge (USDA, 2022).

Through the National Bioenergy Research and Innovation Center (NBERIC) established in 2018, more efforts are being made to realize research and development projects in bioenergy, especially in finding new feedstock to produce more biofuels (USDA, 2024).

Nipa sap is a potential material to be processed into bioethanol (Hidayat, 2018). This potential is highly viable given the natural abundance of the nipa palm (Nypa fruticans) across Philippine estuaries. The country possesses approximately 8,000 hectares of natural nipa stands, making it the third-largest global repository of the species after Indonesia and Papua New Guinea (Centre for Agriculture and Bioscience International [CABI], 2019), with dense localized concentrations capable of sustaining commercial extraction (Mateo et al., 2023). Furthermore, utilizing this abundant resource is highly sustainable. Nipa sap is collected easily by tapping the stalk and cutting off the edible inflorescence of the palm. Harvesting the sap causes no waste and no damage to the environment, and it will have no effect on the continuous growth of the palm (Hidayat, 2018). In 2019, DENR in collaboration with Aklan State University (ASU) launched a project to establish a 5.6-hectare nipa plantation specifically for bioethanol production in Barangay Calizo, Balete, Aklan. The project successfully planted 14,000 seedlings and trained a dedicated association of 14 local planters (ASU, 2019).

To effectively utilize this abundant local feedstock, the fermentation process must be highly efficient. Yeasts are very important in the production of ethanol. However, the problem that has been ravaging ethanol producing industries for decades now is the ability of industrial yeast isolates to withstand ethanol production stress conditions while giving out optimal ethanol yield (Antia, Akan, Stephen, & Eno-ibanga, 2018). These stressors include rise in temperature, ethanol concentration and pH. It not only inhibits yeast growth but also inhibits ethanol productivity, which is a major cause of low ethanol conversion efficiency.

The present study aimed to isolate indigenous yeast strains from naturally fermenting nipa sap and evaluate their potential for bioethanol production through phenotypic characterization and stress tolerance screening. Specifically, the isolates were assessed for their tolerance to elevated temperature, acidic pH, osmotic stress, and ethanol stress, followed by evaluation of their ethanol-producing capability. The fermentation products were further verified using Fourier Transform Infrared (FTIR) spectroscopy. By identifying stress-tolerant indigenous yeast isolates with promising fermentation characteristics, this study contributes baseline information for the future development of locally sourced microbial inoculants for sustainable bioethanol production from nipa palm sap.

2. MATERIALS AND METHODS

2.1 Study Site and Collection of Nipa Sap

Naturally fermenting nipa (*Nypa fruticans* Wurmb.) sap was collected from three sampling sites in Barangay Calizo, Balete, Aklan, Philippines. Sap collection followed the protocol developed by the MMSU–USAID STRIDE Nipa Project (Madigal et al., 2019) with minor modifications. To minimize contamination and delay spontaneous fermentation, sap was harvested using a closed collection system consisting of polyethylene terephthalate (PET) bottles attached directly to the nipa peduncle. The closed collection system preserved the chemical composition of the sap by preventing exposure to the external environment.

Freshly dripping sap was collected continuously for up to 72 h, immediately stored in an ice bath, and transported to the Pharmacy Laboratory of Saint Gabriel College, Kalibo, Aklan for microbiological analysis.

2.2 Isolation and Purification of Yeast Isolates

Yeast isolation was performed following the protocol of Madigal et al. (2019) with minor modifications. Nipa sap samples were serially diluted to 10^{-4} using sterile 0.85% (w/v) sodium chloride solution. Aliquots (1 mL) of the diluted samples were spread onto Potato Dextrose Agar (PDA) supplemented with 100 ppm streptomycin to inhibit bacterial growth. Plates were incubated in an inverted position at 35°C for 3–5 days.

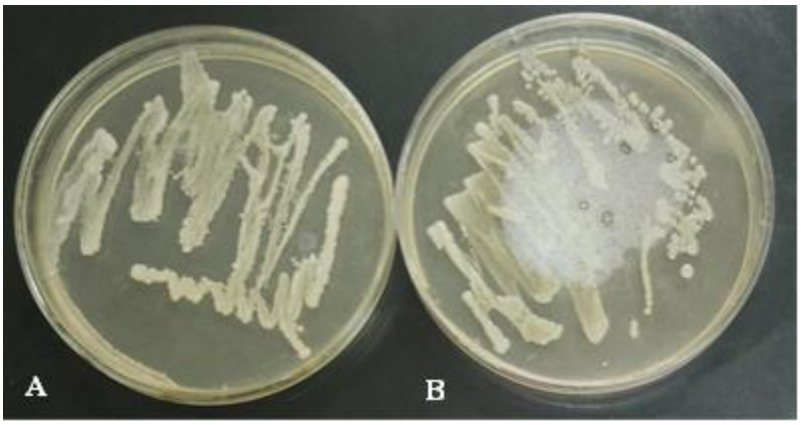
Distinct yeast colonies were selected based on differences in colony morphology, including colony form, color, elevation, margin, texture, and surface characteristics. Selected colonies were repeatedly subcultured on fresh PDA plates until pure cultures were obtained. Pure isolates were maintained on PDA slants at 4°C for short-term storage until further analysis.

2.3 Morphological and Microscopic Characterization

Pure yeast isolates were streaked onto PDA plates and incubated at 35°C for 3–5 days. Colony morphology was evaluated by observing colony color, shape, texture, elevation, and overall appearance.

Microscopic characterization was performed using methylene blue staining. A small amount of each colony was mounted on a clean glass slide, stained with methylene blue, and examined under a light microscope to determine cellular morphology, including cell shape and arrangement.

Figure 1. Macroscopic Morphology and Pure Culture Isolation of the Putative Yeast Isolates from Nipa Sap



Note. The isolates were cultivated on Potato Dextrose Agar (PDA) utilizing the standard streak plate method to isolate distinct colonies. (A) Isolate NY1 and (B) isolate NY2 both demonstrate uniform microbial growth, exhibiting continuous, creamy-white, opaque, and smooth mucoid colonial development along the primary and secondary inoculation lines

2.4 Stress Tolerance Assays

Stress tolerance assays were conducted according to the procedure described by Bondoc (2014), as cited by Madigal et al. (2019). Commercial *Saccharomyces cerevisiae* (brewer's yeast) served as the positive control. Each treatment was performed in five independent replicates.

Colony growth under each stress condition was documented photographically, and colony diameters were measured using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Growth inhibition under increasing stress conditions was used to evaluate stress tolerance.

2.4.1 Thermotolerance

Yeast isolates were inoculated onto PDA plates and incubated at 30, 37, 40, and 45°C. Colony growth after incubation was compared to determine temperature tolerance.

2.4.2 Osmotolerance

Potato Dextrose Agar was supplemented with sucrose at concentrations of 0, 1, 5, 10, 15, and 20% (w/v). Yeast isolates were inoculated onto the prepared media and incubated at 30°C for 48 h before growth assessment.

2.4.3 Ethanol Tolerance

To evaluate ethanol tolerance, PDA plates were supplemented with ethanol concentrations of 0 (control), 2.5, 5, 10, 15, and 20% (v/v). Inoculated plates were incubated at 30°C for 48 h, after which colony growth was measured.

2.4.4 Acid Tolerance

Acid tolerance was evaluated by adjusting PDA medium to pH 2.0, 2.5, 3.0, and 4.0. PDA at its normal pH (6.5) served as the control. Following inoculation, plates were incubated at 30°C for 48 h before colony growth was assessed.

2.5 Evaluation of Ethanol Production

Yeast isolates exhibiting superior performance during stress tolerance screening were selected for ethanol production experiments. Commercial brewer's yeast was included as a reference strain. Two fermentation media were used: Yeast Peptone Dextrose (YPD) broth and Simulated Nipa Sap (SNS) medium.

2.5.1 Inoculum Preparation

Pure cultures maintained on PDA slants were transferred to fresh PDA plates and incubated for 24 h. Two inoculum media were prepared: YPD broth containing 1% yeast extract, 5% dextrose, and 2% peptone, and SNS medium composed of 4% sucrose, 0.45% fructose, 0.35% glucose, and 0.2% maltose. Both media were adjusted to pH 4.5 and sterilized at 121°C (15 psi) for 15 min.

Individual isolates were inoculated into 50 mL of the respective medium contained in 150-mL Erlenmeyer flasks and incubated at 30°C with shaking for 18 h.

2.5.2 Yeast Acclimatization

To minimize osmotic shock, the entire inoculum culture was transferred aseptically into 75 mL of fresh medium containing increased sugar concentrations (10% for YPD and 15% for SNS), resulting in a final working volume of 125 mL. Cultures were incubated at 30°C with shaking for an additional 18 h.

2.5.3 Fermentation

Following acclimatization, cell suspensions were standardized to an optical density (OD_{660}) of 1.0, corresponding to approximately 1×10^8 cells mL^{-1}. A 2% (v/v) inoculum was transferred into fresh fermentation medium containing 10% sugar for YPD or 20% sugar for SNS to obtain a final fermentation volume of 125 mL. Fermentation was carried out at 30°C for 48 h.

2.6 Distillation of Fermentation Broth

Fermented cultures were clarified by filtration prior to distillation. Each clarified broth underwent an initial stripping distillation to concentrate ethanol, followed by fractional distillation using a fractionating column to improve ethanol separation. Distillation was performed using a heating mantle while maintaining the column temperature between 78 and 79°C. The initial head fraction was discarded to eliminate volatile impurities, and collection was terminated before the tails fraction was reached. Distilled samples were collected in sterile containers, and the volume of each distillate was recorded prior to chemical analysis.

2.7 Fourier Transform Infrared (FTIR) Spectroscopy

Distilled fermentation products were analyzed using a PerkinElmer Spectrum IR spectrometer (Version 10.7.2) at the Biotechnology and Biomedical Research Laboratory, West Visayas State University, Iloilo City. FTIR spectroscopy was employed to verify the presence of ethanol by identifying characteristic infrared absorption bands corresponding to ethanol functional groups. Spectra obtained from each sample were compared with the instrument's reference library, and library match scores were recorded. Manual interpretation of diagnostic absorption peaks was also performed to confirm the identity of ethanol.

2.8 Statistical Analysis

Morphological characteristics of the yeast isolates were analyzed descriptively. Quantitative data obtained from the stress tolerance assays were expressed as the mean of five independent replicates. Colony diameters measured using ImageJ were subjected to one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) test to determine significant differences among treatments at a significance level of $p < 0.05$.

FTIR spectra were interpreted using the instrument software by comparing the spectra of each sample with the built-in chemical reference library. Diagnostic absorption peaks were further examined to verify the presence of ethanol in the distilled fermentation products.

3. RESULTS

3.1 Isolation and Morphological Characterization of Indigenous Yeast Isolates

Two indigenous yeast isolates, designated NY1 and NY2, were successfully recovered from naturally fermenting nipa (*Nypa fruticans*) sap collected from two of the three sampling sites in Calizo, Balete, Aklan. Samples from the third site were dominated by rapidly growing filamentous fungi, preventing the isolation of yeast colonies.

Both isolates produced creamy-white to off-white colonies on Potato Dextrose Agar (PDA). Colonies were circular, convex, smooth, opaque, and exhibited entire margins with a characteristic mucoid texture (Table 1; Figure 1). Giant colony cultures developed continuous radial growth from the inoculation point. NY1 formed a central opaque colony with a slightly irregular peripheral margin, whereas NY2 exhibited a similar growth pattern but developed a more pronounced lobate colony margin (Figure 2).

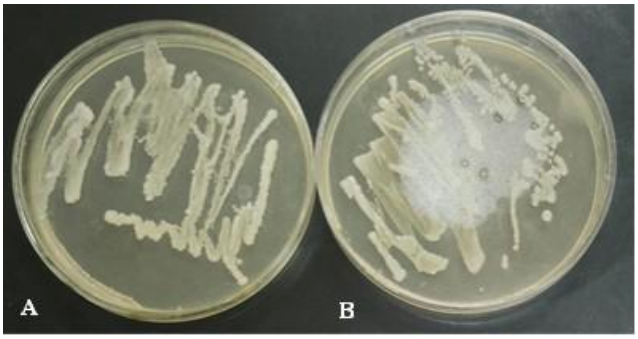
Microscopic examination following methylene blue staining revealed that both isolates consisted of unicellular, ovoid to ellipsoidal cells reproducing by budding (Figure 3). Cells were observed singly, in pairs, or in small clusters, consistent with typical yeast morphology. Most cells remained unstained, indicating high viability.

Two pure cultures of a putative yeast isolate was consistently obtained from the nipa sap samples. The isolation of a single dominant strain could be attributed to the length of natural fermentation prior to isolation, as the samples were collected from 72-hour naturally fermenting nipa sap. In a study by Madigal et al. (2019), Saccharomycodes sp. was observed from 0 h until 48 hours of fermentation but was no longer detectable at 72 hours. Beyond 48 hours, toxic metabolic byproducts accumulate, nutrients deplete, and the environment becomes highly acidic, allowing only highly resistant microorganisms to predominate.

Furthermore, morphological characterization revealed that two of the three independent sampling sites yielded nearly identical isolates. Samples from the third site, however, were overpowered by rapid fungal overgrowth, precluding the isolation and morphological observation of the target strain. This is a well-documented challenge in the modern isolation of wild yeasts from raw environmental and agricultural samples. As emphasized by Grandhay et al. (2024), raw environmental samples naturally harbor highly competitive, fast-growing filamentous fungi that can rapidly overgrow agar plates, physically masking or completely outcompeting slower-growing target yeasts unless specialized chemical fungal inhibitors are utilized.

Despite the contamination at the third site, the uniform recovery of this specific strain, designated temporarily as Nipa Yeast (NY), across the remaining collection sites sharing a common brackish ecological profile in Calizo, Balete, suggests that this particular strain is a highly dominant fermenter in these local Nipa palm stands. As demonstrated by Das and Tamang (2023) in their analyses of naturally fermenting palm saps, the consistent recovery and dominance of specific wild microbial strains across different sampling sites is heavily regulated by shared local agro-climatic factors and the specific geographical profile of the palm environment. Therefore, the uniform recovery of this identical strain across multiple testing sites strongly suggests that it is a highly adapted, ecologically dominant fermenter in these local Nipa palm stands.

Figure 1. Macroscopic Morphology and Pure Culture Isolation of the Putative Yeast Isolates from Nipa Sap

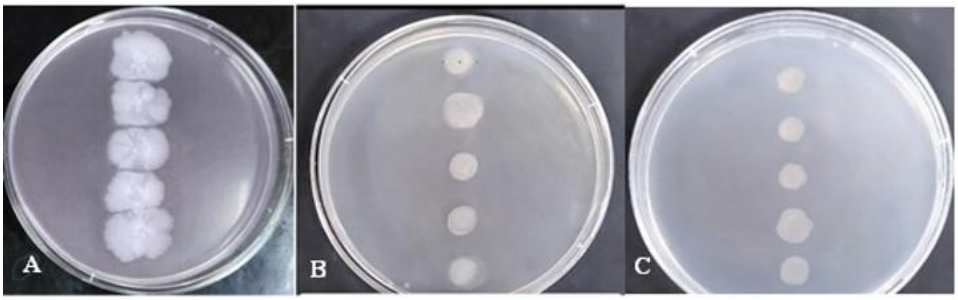


Note. The isolates were cultivated on Potato Dextrose Agar (PDA) utilizing the standard streak plate method to isolate distinct colonies. (A) Isolate NY1 and (B) isolate NY2 both demonstrate uniform microbial growth, exhibiting continuous, creamy-white, opaque, and smooth mucoid colonial development along the primary and secondary inoculation lines

3.2 Stress Tolerance of Indigenous Yeast Isolates

The stress tolerance of NY1 and NY2 was evaluated under ethanol, osmotic, thermal, and acidic conditions and compared with a commercial brewer's yeast (BY). Colony diameters were measured after incubation, and significant differences among strains were determined using one-way ANOVA followed by Tukey's HSD test ($p < 0.05$).

Figure 2. Macroscopic vegetative growth of yeast isolates cultured on potato dextrose agar (PDA) during the 20% ethanol stress evaluation



Note. The images illustrate the five spot-inoculated replicates utilized for quantitative analysis of (A) the commercial Brewer's yeast control, (B) indigenous isolate NY1, and (C) indigenous isolate NY2.

Ethanol tolerance

Both indigenous isolates remained viable across the entire ethanol concentration range (0–20%, v/v). Although the commercial brewer's yeast exhibited significantly greater colony growth at all ethanol concentrations, NY1 and NY2 maintained measurable growth even at 20% ethanol, indicating substantial ethanol tolerance (Table 2).

NY1 produced colony diameters ranging from 8.72 to 10.20 mm, while NY2 ranged from 7.16 to 9.42 mm across the tested ethanol concentrations. The commercial brewer's yeast maintained colony diameters above 14.7 mm under all treatments.

Various studies have shown ethanol tolerance of indigenous strains from nipa sap. A study by Chongkhong and Puangpee (2018) optimized bioethanol production from nipa sap using a yeast strain isolated directly from the fresh sap. Under optimized conditions, this indigenous yeast produced a final ethanol concentration of 113.1 g/L. For a yeast to survive and continue fermentation to this level, it must possess a high ethanol tolerance.

In another study, exploring yeasts from various palm juices, isolates from Nypa fruticans (N3E, N3D, and N1A) were screened for their bioethanol production. Indigenous yeasts produced final ethanol concentrations of 13.2% (v/v), 12.0%, and 12.6%, respectively. This production demonstrates an inherent tolerance to at least 13.2% (v/v) ethanol. The most potent isolate (N3E) was identified as being closely related to Pichia deserticola (Widyaningrum et al., 2022).

Osmotolerance

Increasing sucrose concentration markedly reduced the growth of the commercial brewer's yeast at high osmotic stress. In contrast, both indigenous isolates maintained stable colony growth at 15% and 20% sucrose, where they exhibited significantly larger colony diameters than the commercial control ($p < 0.05$).

At 20% sucrose, NY1 and NY2 produced mean colony diameters of 10.94 and 10.10 mm, respectively, whereas the commercial strain exhibited only 4.09 mm of growth.

Fresh nipa sap is characterized by an extremely high load of sugar. One study assessing the physicochemical properties of fresh sap from Sarawak, Malaysia, measured a total sugar load of 334.2 g/L (approx. 33.4% w/v). The primary sugar was sucrose (231.5 g/L), followed by fructose (42.1 g/L) and glucose (29.7 g/L) (Jaraee et al., 2023). The presence of yeast in this fresh, high-sugar sap confirms their osmotolerant nature, as they must survive this high osmotic pressure to begin fermentation. A

study by Chairul et al. (2020) on bioethanol production on nipa palm referred to as "very high-gravity" (VHG) substrate. The study used Saccharomyces cerevisiae to ferment nipa sap with an initial sugar content of 262.7 g/L. The successful fermentation of this VHG

Thermotolerance

The commercial brewer's yeast maintained significantly larger colony diameters than both indigenous isolates at 30, 37, and 45°C. However, NY1 demonstrated improved growth at 40°C (9.94 mm), becoming statistically comparable to the commercial strain according to Tukey's HSD test. NY2 maintained relatively stable growth between 7.18 and 8.38 mm throughout the tested temperature range.

Other study has found thermotolerant yeasts isolated from nipa. One of which is the P. kudriavzevii isolates that is found to be the most tolerant to high temperatures, with one strain capable of growing at 45°C (113°F). The S. cerevisiae isolates were less tolerant to high heat (Madigal et al., 2019).

Acid tolerance

Both indigenous isolates exhibited significantly greater tolerance to acidic conditions than the commercial brewer's yeast. At pH 2.0, NY1 and NY2 produced colony diameters of 6.72 and 5.84 mm, respectively, compared with only 4.46 mm for the commercial strain.

Similarly, at pH values of 2.5, 3.0, and 4.0, both indigenous isolates consistently exhibited significantly greater colony growth than the commercial control, while no significant differences were observed between NY1 and NY2.

The study Basso et al. (2011) highlights how the Brazilian bioethanol industry successfully isolated and selected indigenous S. cerevisiae strains directly from their industrial fermenters. These indigenous strains were found to be far more tolerant to the combined stresses of acid washing and high temperatures than any commercial strain. In another study by Madigal et al (2019), P. kudriavzevii isolate was able to tolerate and grow in an extremely acidic environment of pH 2.0. This level of acid tolerance is exceptional and significantly higher than that of many conventional S. cerevisiae strains, which are often inhibited below pH 3.0.

Overall, the indigenous isolates displayed superior tolerance to osmotic and acidic stress, whereas the commercial brewer's yeast demonstrated greater tolerance to ethanol and elevated temperatures.

Table 1. *Stress Tolerance Profiles of Nipa Yeast (NY) and Brewer's Yeast (BY)*

Stress Factor Isolate	Parameters	Growth of Yeast Colony*					
		Brewer's Yeast		NY1		NY2	
Ethanol		**Mean**	**SD**	**Mean**	**SD**	**Mean**	**SD**
	0.0%	15.79[a]	1.08	10.20[b]	1.71	9.42[b]	0.69
	2.5%	15.48[a]	1.58	9.01[b]	1.38	7.98[b]	0.46
	5.0%	15.53[a]	0.24	9.16[b]	0.64	8.36[b]	0.80
	10.0%	15.33[a]	1.76	9.55[b]	1.73	7.16[c]	0.25
	15.0%	15.64[a]	1.83	8.72[b]	1.04	7.74[c]	0.47
	20.0%	14.76[a]	1.08	9.03[b]	1.04	8.26[b]	1.05
Osmotolerance							
	0.0%	14.07[a]	1.42	10.05[b]	3.21	8.92[b]	1.82
	1.0%	12.90	2.63	10.22	3.48	9.04	1.14
	5.0%	9.87	1.38	9.68	0.89	8.84	1.22

	10.0%	9.61	1.23	10.73	2.95	9.26	1.22
	15.0%	4.21[b]	0.41	11.36[a]	2.88	9.88[a]	0.69
	20.0%	4.09[b]	0.21	10.94[a]	1.98	10.1[a]	0.93
Thermotolerance (^{0}C)							
	30^{0}C	15.37[a]	1.90	6.27[b]	1.34	7.18[b]	0.30
	37^{0}C	14.41[a]	1.54	8.37[b]	0.44	7.2[b]	0.35
	40^{0}C	14.36[a]	4.97	9.94[ab]	1.32	8.16[b]	0.42
	45^{0}C	13.48[a]	1.91	9.07[b]	1.10	8.38[b]	0.55
Acid Tolerance							
	2	4.46[b]	0.33	6.72[a]	0.97	5.84[a]	0.45
	2.5	4.34[b]	0.37	8.10[a]	0.72	7.54[a]	0.80
	3	4.34[b]	0.31	7.74[a]	0.55	7.96[a]	0.59
	4	4.37[b]	0.47	8.73[a]	1.63	8[a]	0.35

** Values represent the mean growth. Different superscript letters within the same row indicate statistically significant differences between the strains according to One-Way ANOVA and Tukey's HSD post-hoc test ($p < 0.05$).*

Comparative FTIR Analysis of the Commercial Brewer's Yeast Control

To provide a comparative reference, the commercial brewer's yeast (BY) was fermented under the same conditions in both Yeast Peptone Dextrose (YPD) and Simulated Nipa Sap (SNS) media. The resulting distillates were analyzed using FTIR spectroscopy and compared with the reference spectrum of pure ethanol.

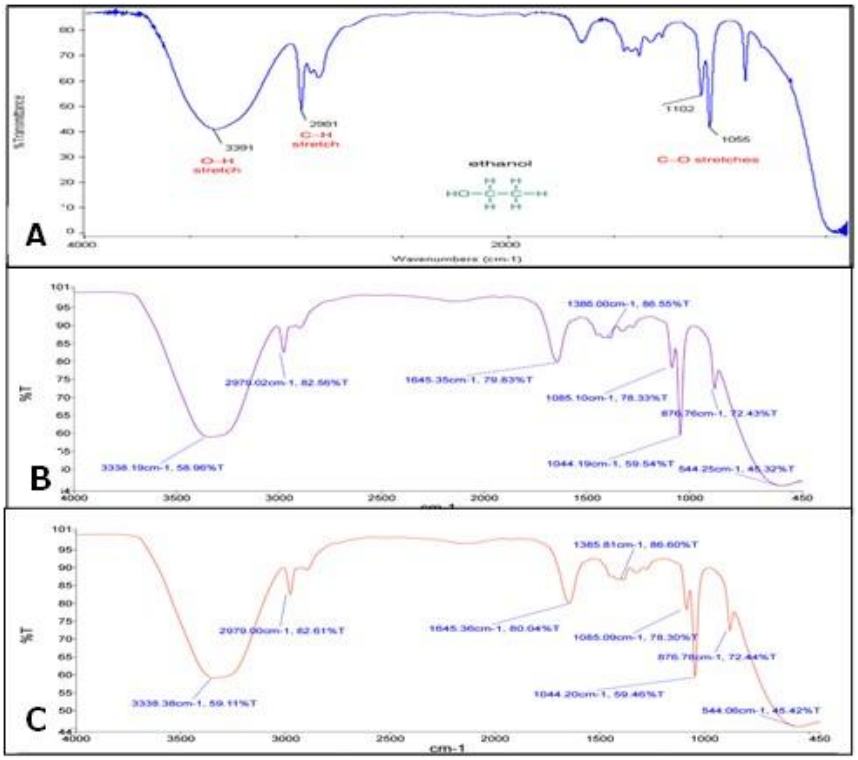
Unlike the indigenous isolates (NY1 and NY2), the FTIR spectra obtained from the brewer's yeast distillates did not exhibit the characteristic spectral profile of ethanol (Figure 6). Although broad O–H stretching bands were observed near 3300 cm^{-1} together with C–H stretching vibrations between 2800 and 3000 cm^{-1}, the fingerprint region differed markedly from the ethanol reference spectrum.

Specifically, the diagnostic double C–O stretching bands characteristic of ethanol (approximately 1045 and 1085 cm^{-1}) were absent. Instead, the spectra displayed a dominant absorption band centered near 1030 cm^{-1}, together with a water-associated H–O–H bending band around 1640 cm^{-1}. These observations indicate that the chemical composition of the distillate differed from that of the ethanol reference under the analytical conditions employed.

The highly similar FTIR profiles obtained from brewer's yeast fermented in both YPD and SNS media suggest that this atypical spectral pattern was reproducible across fermentation media. However, FTIR spectroscopy alone cannot unequivocally identify the compounds responsible for the observed absorption bands. Therefore, the spectra are interpreted as representing an atypical FTIR profile that did not match the ethanol reference spectrum, rather than confirming the presence of a specific alcohol or fermentation product.

Because the objective of this study was to verify ethanol production by the indigenous nipa yeast isolates, subsequent analyses focused on NY1 and NY2, both of which exhibited FTIR spectra that closely matched the characteristic functional groups of ethanol. Definitive identification of the compounds present in the brewer's yeast distillates would require complementary analytical techniques with higher chemical specificity, such as gas chromatography coupled with flame ionization detection (GC-FID) or gas chromatography–mass spectrometry (GC-MS).

Figure 3. Comparison of FTIR spectra of NY1 and NY2 fermented in Simulated Nipa Sap (SNS)



Note. (A) Standard reference spectrum of pure ethanol highlighting key functional group stretching frequencies (O-H, C-H, and C-O) (University of Colorado, n.d.). (B) FTIR spectrum of the fermented yeast distillate for NY1 in SNS, and (C) FTIR spectrum of the fermented yeast distillate for NY2 in SNS showing matching functional group peaks and an additional H-O-H bend indicating the presence of water

Ethanol Production by Indigenous Yeast Isolates

Ethanol productivity was evaluated by measuring the volume of distillate obtained after fermentation in YPD and simulated nipa sap media (Table 5).

In YPD medium, NY1 produced an average of 4.20 mL of ethanol, corresponding to a yield of 3.36% (v/v), whereas NY2 produced 3.80 mL (3.04% v/v). The overall mean ethanol production for YPD cultures was 4.00 mL (3.20% v/v).

Higher ethanol production was observed in simulated nipa sap. NY1 achieved the highest ethanol yield of the study, producing 4.57 mL (3.66% v/v), while NY2 produced 4.10 mL (3.28% v/v). The overall average ethanol production in simulated nipa sap reached 4.33 mL, equivalent to 3.46% (v/v).

Across both fermentation media, NY1 consistently outperformed NY2, and both isolates produced greater ethanol yields in simulated nipa sap than in YPD medium.

Pangastuti et al. (2022) investigated the bioprospecting of ethanol-producing yeasts, isolating strains using YPD agar and evaluating their fermentation capacity in YPD broth. The study confirmed that standardizing the carbon and nitrogen sources through YPD allows for the accurate baseline measurement of ethanol yields (measured via HPLC) before testing alternative or industrial substrates. Their findings support the use of YPD as a highly reliable control medium for observing optimal yeast growth and ethanol conversion.

CONCLUSIONS

This study demonstrated that naturally fermenting nipa (*Nypa fruticans*) sap collected from Calizo, Balete, Aklan is a viable source of indigenous yeast strains with characteristics desirable for bioethanol production. Two isolates (NY1 and NY2) were successfully recovered and exhibited colony morphology and microscopic features consistent with yeast.

Stress tolerance screening revealed distinct physiological characteristics of the indigenous isolates. Both NY1 and NY2 exhibited superior osmotolerance and acid tolerance compared with the commercial brewer's yeast, maintaining growth under high sucrose concentrations (up to 20%) and strongly acidic conditions (pH 2.0–4.0). Although the commercial strain displayed greater overall ethanol tolerance and thermotolerance, NY1 maintained comparable growth at 40°C, suggesting its suitability for fermentation processes where moderate heat stress occurs.

FTIR analysis confirmed the production of ethanol-associated functional groups in distillates obtained from both indigenous isolates following fermentation in YPD and simulated nipa sap (SNS). The characteristic O–H, C–H, and C–O absorption bands closely matched those of the ethanol reference spectrum, confirming the ethanologenic capability of both isolates. Higher ethanol yields were obtained in SNS than in YPD, indicating that the indigenous isolates are better adapted to substrates resembling their natural ecological environment. Among the isolates, NY1 consistently produced the highest ethanol yield.

Overall, the findings indicate that indigenous nipa yeasts, particularly isolate NY1, possess desirable physiological traits for bioethanol production from nipa sap and represent promising candidates for further development in sustainable bioethanol fermentation.

The superior osmotolerance and acid tolerance exhibited by the indigenous isolates suggest that they are well suited for fermentation processes involving high-sugar substrates such as nipa sap. These physiological characteristics may permit fermentation under conditions that naturally suppress bacterial contamination while reducing the need for extensive pH adjustment and sterilization, thereby improving process efficiency.

The higher ethanol production observed in simulated nipa sap compared with conventional YPD medium further suggests that indigenous yeasts are metabolically adapted to the carbohydrate composition of nipa sap. This adaptation may improve fermentation performance when locally available nipa resources are used as feedstock.

The consistent recovery of similar yeast isolates from multiple sampling sites also demonstrates that naturally fermenting nipa sap represents a valuable source of stress-tolerant indigenous microorganisms for future bioprospecting and industrial screening programs.

Collectively, these findings support the potential utilization of locally adapted yeast strains as alternatives or supplements to commercial fermentation cultures for nipa-based bioethanol production.

Study Limitations

This study has several limitations. Identification of the indigenous isolates was based primarily on cultural and microscopic characteristics, and molecular identification was not performed. Consequently, the taxonomic identity of NY1 and NY2 remains unresolved.

The ethanol product was verified using FTIR spectroscopy, which confirms the presence of characteristic ethanol functional groups but does not provide quantitative chemical composition or distinguish all volatile compounds with the specificity achievable using chromatographic techniques such as GC-FID or GC-MS.

Furthermore, fermentation experiments were conducted at laboratory scale using batch cultures. Therefore, the observed stress tolerance and ethanol productivity should be validated under pilot-scale and industrial fermentation conditions.

Recommendations for Future Research

Future investigations should identify NY1 and NY2 using molecular techniques, such as ITS rDNA sequencing or whole-genome sequencing, to establish their taxonomic identity and facilitate comparison with other industrial yeast strains.

Additional studies employing GC-FID, GC-MS, or HPLC are recommended to accurately quantify ethanol concentration and characterize other fermentation products. Optimization of fermentation variables, including inoculum size, temperature, pH, fermentation duration, and substrate concentration, could further improve ethanol productivity.

Finally, pilot-scale fermentation trials using fresh nipa sap should be conducted to evaluate the industrial applicability, process stability, and economic feasibility of these indigenous isolates for sustainable bioethanol production.

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