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Isolation of Novel Yeast from Coconut (*Cocos nucifera* L.) Water and Phenotypic Examination as the Potential Parameters in Bioethanol Production

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Abstract: Yeast is a fermentation agent for producing bioethanol as an environmentally friendly alternative energy. Therefore, this study aims to find novel yeasts with the capability to persevere under acidic, high temperature, and high sugar content conditions, which are required in the bioethanol industry. The yeasts were isolated and identified from coconut (*Cocos nucifera* L.) water by a DNA sequencing method and phenotypic test. Yeast isolation has been completed with a serial dilution procedure and purification was conducted with HiPurA Genomic DNA Purification Spin Kits, which were analyzed by DNA Sequencing. The phenotypic test was carried out with thermotolerant (30 °C and 41 °C), high acidity (lactic acid), and sugar content (molasses 35 °brix) parameters in the media as the initial step of yeast ability screening. Based on the results, the three species of *Candida tropicalis* K5 (*Candida tropicalis* strain L2), K15 (*Candida tropicalis* strain MYA-3404), and K20 (*Candida tropicalis* strain Y277) obtained met the phenotypic standards. This showed that the yeasts have the potential to produce molasses-based bioethanol.

Keywords: candida; coconut water (*Cocos nucifera* L.); phenotypic; DNA sequencing

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1. Introduction

Yeast is a unicellular microorganism in the biotechnology process that plays an important role in bioconversion activity for the production of alcoholic products such as wine, pickles, beer, and bread. The wide applications of yeast make it possible for it to be used in bioethanol fermentation. There are various kinds of raw materials for bioethanol production including lignocellulosic biomass [1], molasses [2], and agriculture waste [3]. Meanwhile, molasses is an advantageous source of fermentable sugar because it contains 48–55% sucrose. During its fermentation, yeast is usually added to convert the sucrose content into bioethanol through its enzymatic metabolism [4].

The effective and efficient exploration of yeast in the production of bioethanol from molasses is always challenging. The novelty of this study is discovering novel yeast in coconut (*Cocos nucifera* L.) water that can get through stress tolerance. Several parameters have shown successful bioethanol processing such as thermotolerance, acid resistance, and

sugar tolerance. This stress tolerance is important in yeast screening, and it is commonly referred to for phenotypic identification.

The discovery of yeast that meets phenotypic standards can be carried out through isolation. Previous studies showed that indigenous yeast isolation from a certain source is a reliable method to discover a novel product with a specific ability and a significant effect on the yield of bioethanol [5]. Coconut water was selected as the source of yeast because it contains many nutrients needed by cells, which are sugar in the form of sucrose, as well as amino and organic acids [6]. Saraswati (2014) tested the effect of coconut water on the growth of *Saccharomyces cerevisiae*, with the hypothesis being accepted [7]. The presence of nutrients and compounds possessed by coconut water can be the first step in characterizing and identifying microorganisms from coconut water. The identification of microorganisms in coconut water is expected to uncover better novel yeast compared to *Saccharomyces cerevisiae* in biotechnology exploration.

Saccharomyces cerevisiae is not resistant to the high concentrations of ethanol produced [8]. Its optimum activity occurs at 28–35 °C and 3.5–6.0 pH [9]. Meanwhile, the bioethanol industry runs the production in a reactor that uses a high temperature inlet with a huge amount of molasses. In conclusion, the purpose of this study is to examine the phenotypic responses of yeast isolated from coconut water by observing the feedback of non-saccharomyces yeast on high temperature, acidity, and sugar concentration (°brix) in the growing medium. Yeast with a higher probability of stress tolerance was selected as the novel yeast.

2. Materials and Methods

2.1. Sample Preparation

Coconut (*Cocos nucifera* L.) was obtained from a traditional market in Ogan Ilir Regency, South Sumatra, Indonesia. The coconut was peeled, and the obtained water was transferred to a sterile Erlenmeyer for further processing.

2.2. Yeast Isolation from Coconut Water (Cocos Nucifera L.)

A total of 10 mL of the sample was diluted using a serial dilution procedure (10^{-1}–10^{-5}). The yeast population was carried out through the modification of Maciel et al.'s [10] method by taking aliquots (0.1 mL) of the serial dilution and spreading it on Yeast Malt Agar (YMA) with 2 g glucose, 2 g peptone, 1 g malt extract, 1 g yeast extract, 2 g agar, plus chloramphenicol at 100 mg per 100 mL of distilled water. The samples were incubated at 30 °C for 3 days, and the obtained yeast cultures were stored in 20% glycerol stock for further identification.

2.3. Extraction of Yeast DNA Genome

DNA purification was conducted using HiPurA Genomic DNA Purification Spin Kits, which provide a quick and easy method for application in PCR (Polymerase Chain Reaction) based on the manufacturer's procedures.

2.4. DNA Sequencing

DNA sequences were amplified using two common primers, namely ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The amplification was carried out through a reaction mixture containing 1 × 25 µL (9.5 sterile water, 12.5 MyTaq Red Mix, 10 M ITS1, 10 M ITS4, and 1 DNA template). Amplicons were amplified according to a previous method under PCR conditions of 95 °C for 3 min (initial denaturation), continued (95 °C, denatured 10 s at 95 °C, annealing 30 s at 52 °C, extension at 72 °C for 45 s) at 35 cycles, with a final extension at 72 °C for 5 min. Subsequently, the PCR product was electrophoresed using 1% agarose gel. Sequencing data were taken from the National Center for Biotechnology Information NCBI/BLAST (blast.ncbi.nlm.nih.gov/BLAST.cgi) to create a phylogenetic tree based on the neighbor-joining algorithm.

2.5. Phenotypic Test

The isolated yeast was grown in YPDA (Yeast Peptone Dextrose Agar) at a pH of 3.5 and in YPA-Molasse 3 °brix media at temperatures of 30 °C and 41 °C. The pH of 3.5 was made by adding lactic acid to YPDA media until the pH value was obtained. Meanwhile, YPA-molasses 35 °brix was made by adding pretreated 35 °brix molasses into YPA media. The growth of yeast was observed and compared to control Saccharomyces cerevisiae on each representative medium after 24 h of incubation time.

3. Results

Nucleic acid (Genomic DNA) quantification was completed between 3.80–79.90 ng/µL (Table 1), while sequencing produced a specific ratio of $A_{260/280}$ and $A_{260/230}$ through PCR amplification and bi-directionality. The ratio resulted from the uv-vis spectrum ranging from 1.63–5.35 and 0.48–2.01, respectively. Therefore, the absorbance scale indicated the DNA purity, and ≥1.8 signified a pure DNA sample.

Table 1. The genomic DNA concentration of the yeast isolates.

Isolate	Concentration (ng/µL)	$A_{260/280}$	$A_{260/230}$
K1	8.20	2.09	0.77
K2	18.60	2.08	1.20
K3	45.10	2.28	2.01
K4	8.00	2.01	0.81
K5	10.30	2.16	1.01
K6	3.80	1.63	0.48
K7	36.50	2.25	1.52
K8	79.90	1.84	0.60
K9	33.50	1.94	0.60
K10	59.80	2.34	2.03
K11	43.40	1.85	0.49
K12	7.00	2.28	0.97
K13	17.70	2.12	1.11
K14	4.80	1.94	0.62
K15	11.10	1.86	0.73
K16	4.30	5.35	0.48
K17	5.00	1.64	0.52
K18	5.10	1.77	0.57
K19	7.10	2.98	0.92
K20	7.00	1.64	0.48

Figure 1 shows the PCR result, which was amplified and assessed by electrophoresis, and the band on each DNA fragment describes the purity of the gene. Based on the photo, the 20 samples (K1–K20) read around 500–900 base pairs. All the isolates have a single band, with the exception of isolates K4 and K12, which have two bands. The band below describes the total impurities.

Figure 2 shows the phylogenetic tree of isolates K1, K2, K3, K4, and K5. Meanwhile, K1 was identified as *Henseniaspora opuntiae* strain NS02 (KT226114.1) with a 99.86% similarity of identity. *Henseniaspora meyeri* CBS:8775 (KY103531.1) was identical with isolates K2 and K3 as well as having a 99.81% identity percentage. Furthermore, isolate K4 was analogous with *Meyerozima carpophila* strain CBS5256 (MK394110.1) with a 99.83% resemblance, while K5 was *Candida tropicalis* strain L2 (MK752673.1) because it was comparable with a 99.81% identity percentage. The phylogenetic tree of isolates K6, K7, K8, K9, and K10 is shown in Figure 3. It was discovered that isolate K6 had a 100% similarity of identity with *Henseniaspora opuntiae* strain F173 (KY497945.1). Meanwhile, isolates K7 and K8 were both equally interpreted as *Henseniaspora meyeri* culture CBS:8775 (KY103531.1) with a 100% identity percentage. An isolate was classified as *Saccharomyces cerevisiae* strain KSD-Yc (CP024006.1), which was 100% identical to isolate K9. *Henseniaspora meyeri* culture CBS:8775 (KY103531.1) was described as isolate K10 with a 100% resemblance.

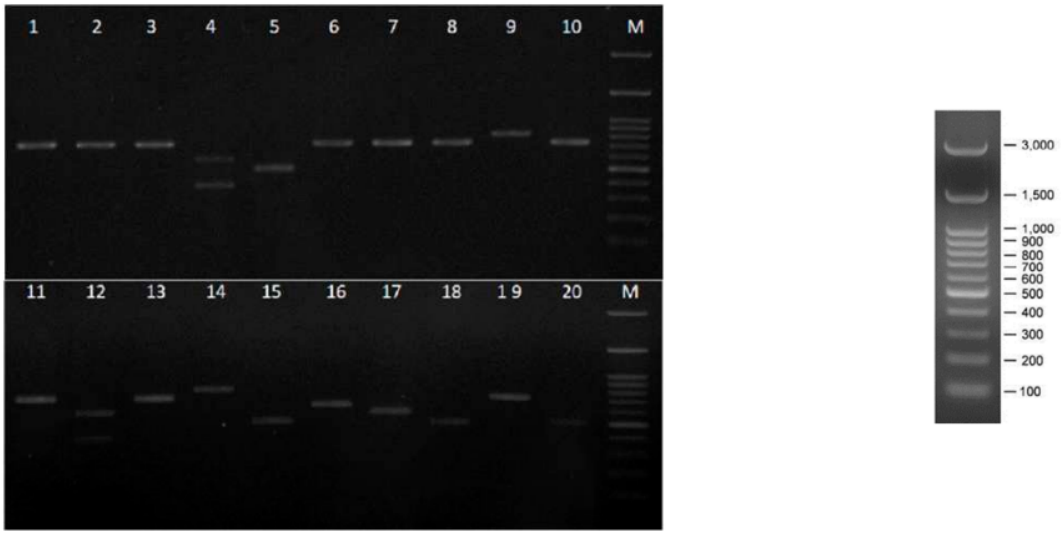


Figure 1. Gel photo of the isolate as a PCR product in an electrophoresis gel.

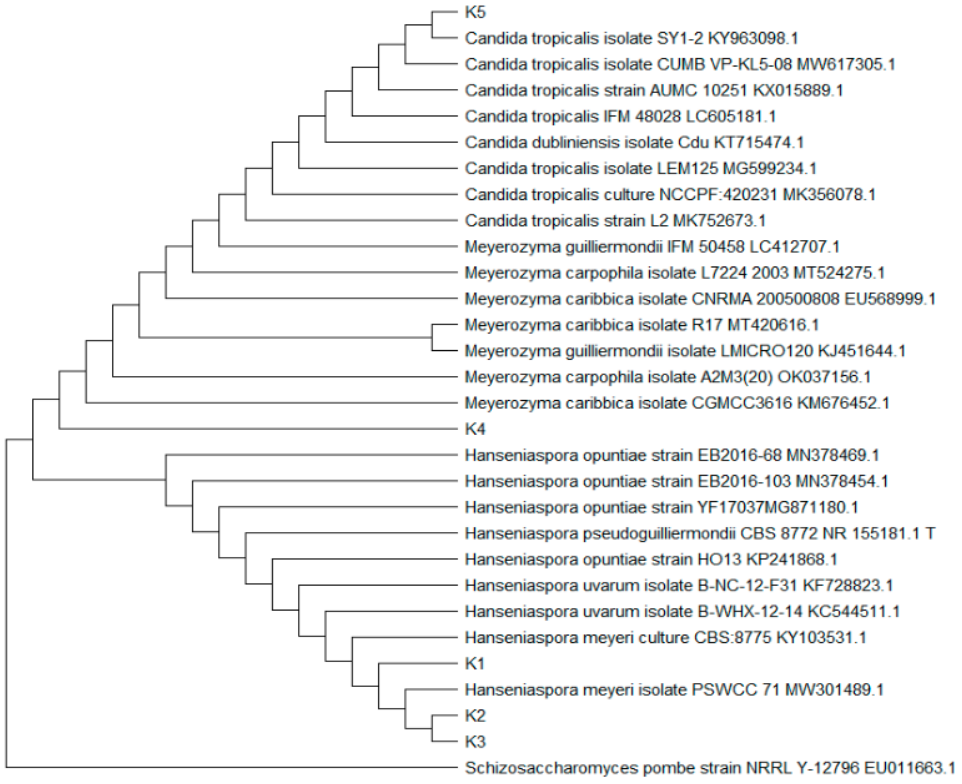


Figure 2. Dendrogram of yeast isolates K1, K2, K3, K4, and K5.

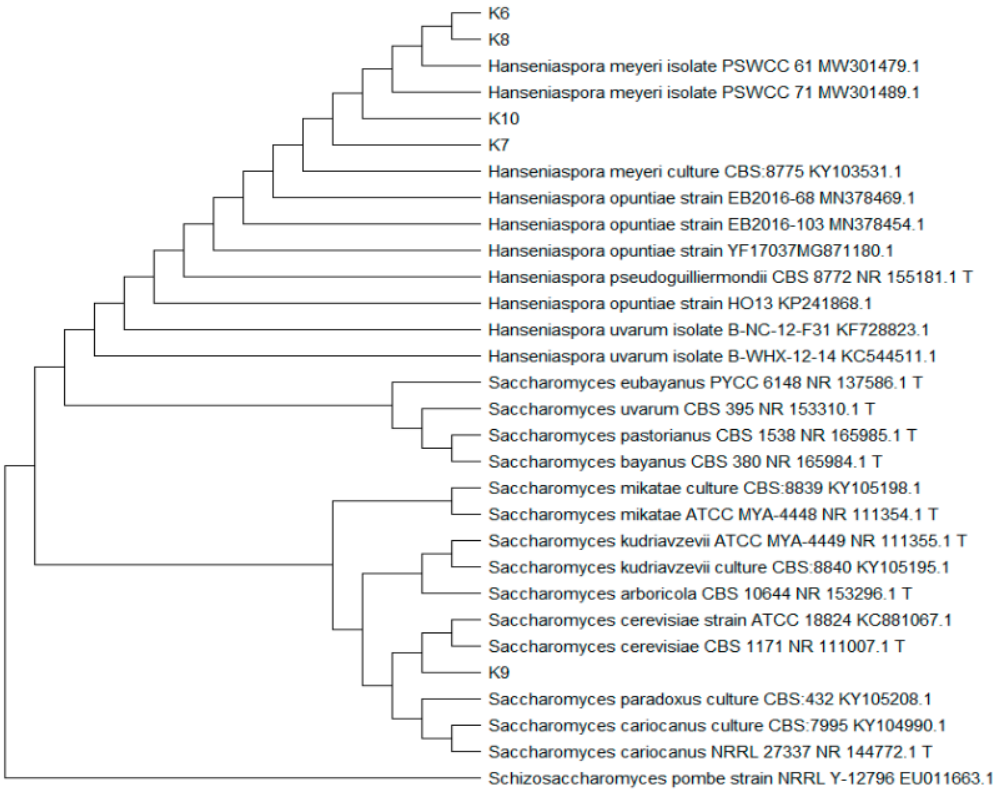


Figure 3. Dendrogram of yeast isolates K6, K7, K8, K9, and K10.

Figures 4 and 5 show the phylogenetic tree of isolates K11, K12, K13, K14, K15, K16, K17, K18, K19, and K20. *Henseniaspora thailandica* (AB501145.1) was discovered as isolate K11 with a 100% identity percentage, while isolate K12 was 99.52% identical with *Meyerozima carribica* Strain UFLA CWFY11 (KM402049.1). *Henseniaspora meyeri* culture CBS:8775 (KY103531.1) was transcribed as K13 with a 99.81% similarity of identity. Isolate K14 was also considered to be *Saccharomyces cerevisiae* strain KSD-Yc (CP024006.1), like K9, with a 100% identity percentage. Furthermore, K15 was transcribed as *Candida tropicalis* strain MYA-3404 (CP047875.1) with a 99.61% identity percentage, while *Lachance fermentati* strain CNRMA8.216 (KP132361.1) was isolate K16 with a 99.70% identity similarity. The isolate K17 was found to be *Meyerozima carribica* strain CBS 5256 (MK394110.1) at 100%, while K18 resembled *Candida othopsilosis* (FM178396.1), which had a 100% identity percentage. *Henseniaspora ovarum* culture CBS:2580 (KY103573.1) was similar to isolate K19, at 99.91%. Another *Candida tropicalis* strain Y277 (KT459476.1) was also discovered in isolate K20, which was 100% identical.

The phenotypic identification was carried out at two different temperatures for the comparison, namely 30 °C and 41 °C. The 20 isolates were compared to *Saccharomyces cerevisiae* (X) in various growing media, which included YPDA, pH 3.5 (by adding lactic acid), and YPA-Molasses 35 °brix. Figures 6–8 (a) are the visualization of the growth after 24 h of incubation at 30 °C and Figures 6–8 (b) at 41 °C. The result showed that isolates K5, K15, and K20 had constant growth during the observations.

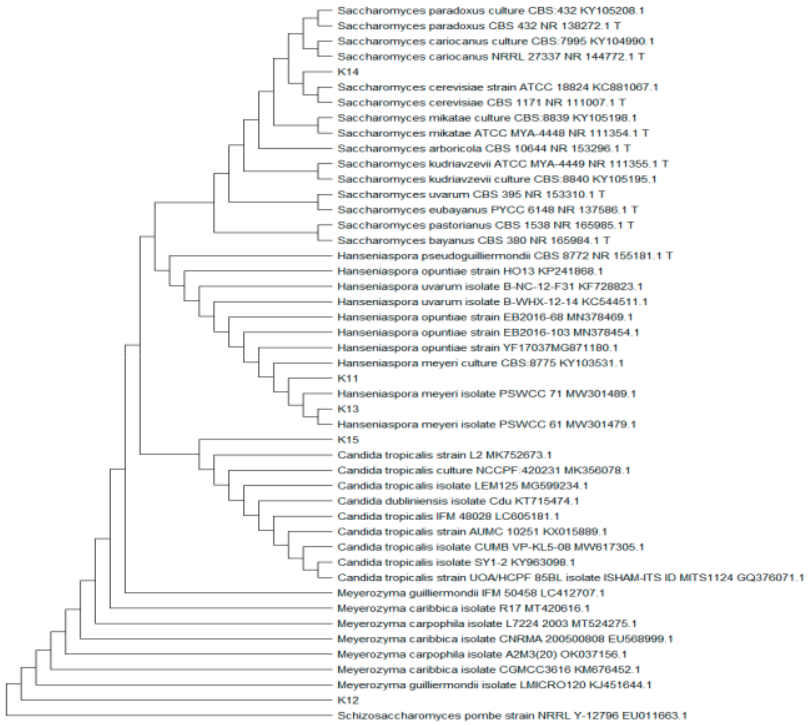


Figure 4. Dendrogram of yeast isolates K11, K12, K13, K14, and K15.

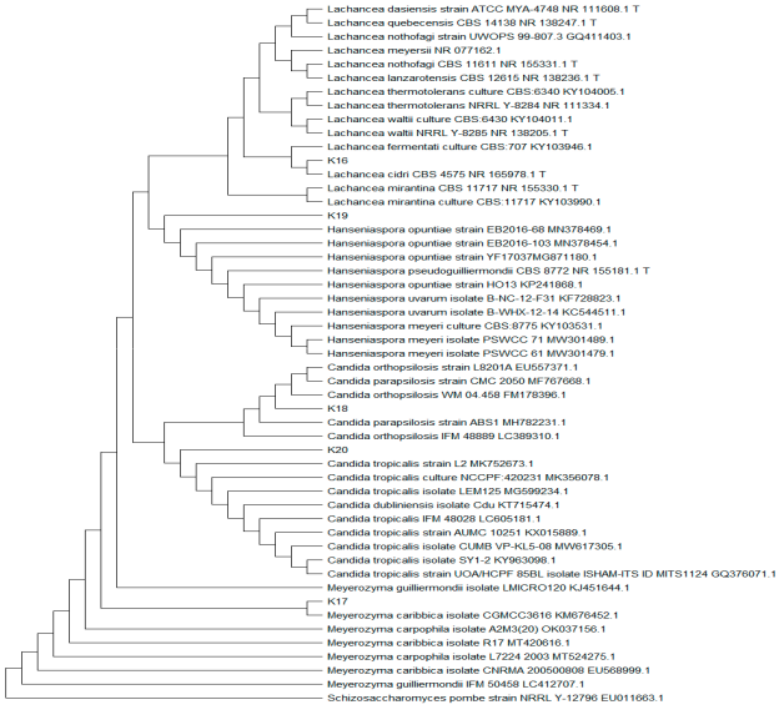
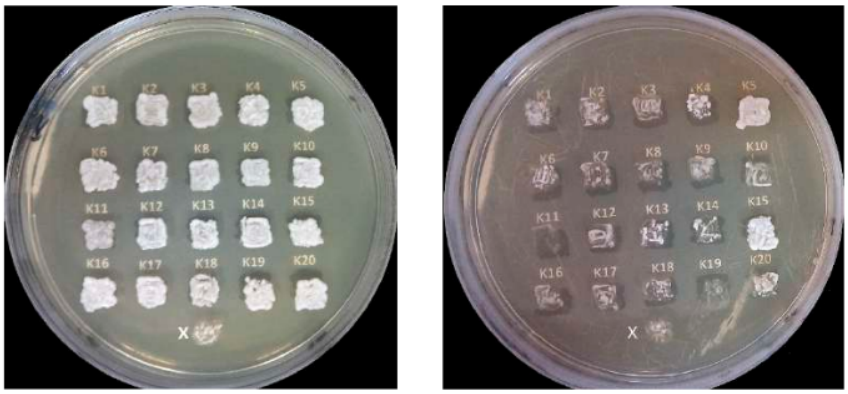
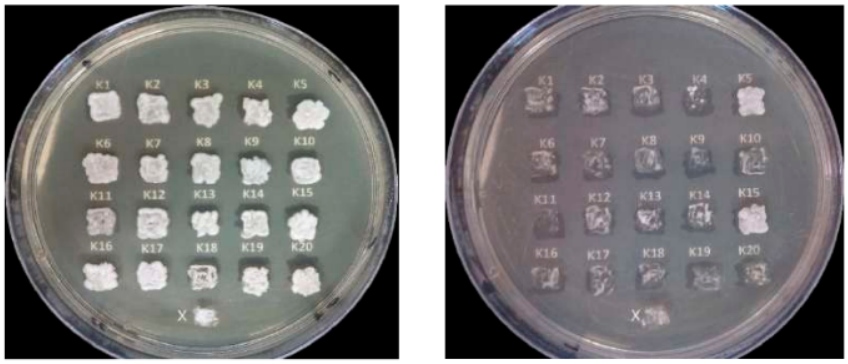


Figure 5. Dendrogram of yeast isolates K16, K17, K18, K19, and K20.



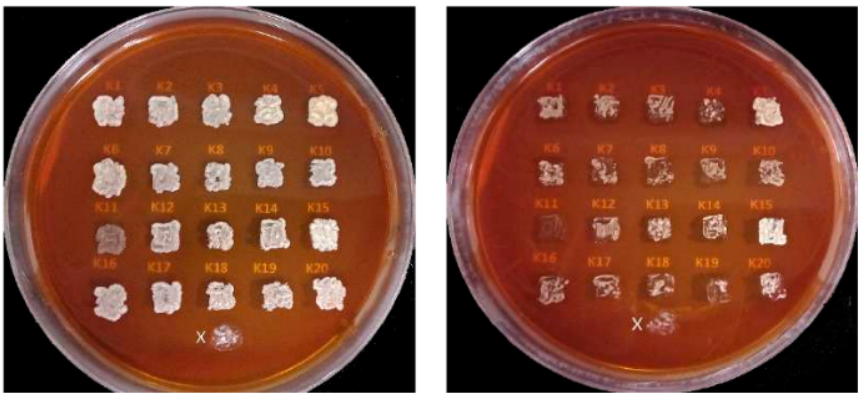
(**a**) (**b**)

Figure 6. The growth of 20 isolates and X (Saccharomyces cerevisiae) in YPDA at (**a**) 30 °C and (**b**) 41 °C.



(**a**) (**b**)

Figure 7. The growth of 20 isolates and X (Saccharomyces cerevisiae) in YPDA pH 3.5 at (**a**) 30 °C and (**b**) 41 °C.



(**a**) (**b**)

Figure 8. The growth of 20 isolates and X (Saccharomyces cerevisiae) in YPA-Molasses 35 °brix at (**a**) 30 °C and (**b**) 41 °C.

4. Discussion

Microorganisms play an important role in fermentation. The fermentation process depends on the isolation/development of yeast, which can ferment various type of sugars. In order to achieve this goal, investigations were carried out to obtain indigenous yeast from various sources such as fermented natural ingredients [11], fruit skins [12], fruits [13], and coconut water. The identification of yeast in coconut (*Cocos nucifera* L.) water was expected to contribute to the genetic diversity, with a significant potential for bioethanol production. There are limited investigations of coconut water that focus on this because the majority of the research focuses only on the specific use of biochemical composition and preservation techniques.

Coconut water contains minerals, a high sugar content, acidity, protein, crude fat, and total phenolic compounds [14]. Furthermore, the sugar content is 80% sucrose, 3% glucose, and 7% fructose, with a low glycemic index (35–54) [15]. The nutrients and other compounds in coconut water can be the initial step in characterizing and identifying the existence of indigenous yeast that is needed for living.

In this study, 20 isolates were obtained from coconut water. Based on the identification result in Table 2, a total of 14 different indigenous yeast strains were discovered among the isolates. These include K1 (*Henseniaspora opuntiae* strain NS02), K2-K3-K7-K8-K10-K13 (*Henseniaspora meyeri* culture CBS:8775), K4 (*Meyerozima carpophila* strain CBS5256), K5 (*Candida tropicalis* strain L2), K6 (*Henseniaspora opuntiae* strain F173), K9-K14 (*Saccharomyces cerevisiae* strain KSD-Yc), K11 (*Henseniaspora thailandica)*, K12 (*Meyerozima carribica* Strain AUMC 7262), K15 (*Candida tropicalis* strain MYA-3404), K16 (*Lachance fermentati* strain CNRMA8.216), K17 (*Meyerozima carribica* strain CBS 5256), K18 (*Candida othopsilosis)*, K19 (*Henseniaspora ovarum* culture CBS:2580), and K20 (*Candida tropicalis* strain Y277). It was necessary to test the obtained group of yeasts to examine their potential for surviving under stress control by a phenotypic test as the first testing step through selective media and temperatures.

Table 2. The summary of BLAST-N 16 rRNA genes of yeast isolates.

Isolate	Accession Number	Species	Max Score	Total Score	Query Coverage	E Value	Percent Identity
K1	KT226114.1	*Henseniaspora opuntiae* strain NS02	1351	1351	99%	0.0	99.86%
K2	KY103531.1	*Henseniaspora meyeri* culture CBS:8775	959	959	100%	0.0	99.81%
K3	KY103531.1	*Henseniaspora meyeri* culture CBS:8775	952	952	100%	0.0	99.81%
K4	MK394110.1	*Meyerozima carpophila* strain CBS5256	1083	1083	99%	0.0	99.83%
K5	MK752673.1	*Candida tropicalis* strain L2	898	898	100%	0.0	99.81%
K6	KY497945.1	*Henseniaspora opuntiae* strain F173	935	935	100%	0.0	100%
K7	KY103531.1	*Henseniaspora meyeri* culture CBS:8775	957	957	100%	0.0	100%
K8	KY103531.1	*Henseniaspora meyeri* culture CBS:8775	953	953	99%	0.0	100%
K9	CP024006.1	*Saccharomyces cerevisiae* strain KSD-Yc	1520	1520	99%	0.0	100%
K10	KY103531.1	*Henseniaspora meyeri* culture CBS:8775	957	957	100%	0.0	100%
K11	AB501145.1	*Henseniaspora thailandica*	1347	1347	97%	0.0	100%
K12	KM402049.1	*Meyerozima carribica* Strain UFLA CWFY11	1105	2215	100%	0.0	99.51%
K13	KY103531.1	*Henseniaspora meyeri* culture CBS:8775	952	952	99%	0.0	99.81%
K14	CP024006.1	*Saccharomyces cerevisiae* strain KSD-Yc	1515	10,502	100%	0.0	100%
K15	CP047875.1	*Candida tropicalis* strain MYA-3404	948	2845	99%	0.0	99.61%
K16	KP132361.1	*Lanchance fermentati* strain CNRMA8.216	1221	1221	99%	0.0	99.70%
K17	MK394110.1	*Meyerozima carribica* strain CBS 5256	1123	1123	99%	0.0	100%
K18	FM178396.1	*Candida othopsilosis*	948	948	99%	0.0	100%
K19	KY103573.1	*Henseniaspora ovarum* culture CBS:2580	1312	1312	99%	0.0	99.91%
K20	KT459476.1	*Candida tropicalis* strain Y277	974	974	100%	0.0	100%

A phenotypic test was carried out under some stressful conditions to discover yeast with a superior strain. The conditions were thermotolerance, acidity tolerance and a high

molasses content. The phenotypic scheme was relevant for bioethanol production based on the result in Table 3 and Figures 6–8. On an industrial scale, bioethanol production usually takes place at 41 °C [16] because the fermentation reactor sets a high energy intake [17]. Due to this reason, the temperature setting was 30 °C and 40 °C. Bioethanol was produced in an anaerobic and acidity setup, while pH 3.5 was expected as the maximum acid environment for yeast to convert the sugar into ethanol [18,19]. It was discovered that yeast worked sensitively at a certain maximum sugar content concentration based on the raw material [20, 21]. The analysis of the physicochemical characteristics of molasses with 48–54% sucrose shows the potential for producing alcohol products [22] because it is fermentable without modification. Based on this fact, the investigation of high sugar content was completed in yeast agar molasses media with a 35 °brix concentration. Therefore, the phenotypic landscape was completed by comparing the superiority between *Saccharomyces cerevisiae* and indigenous yeast from coconut water.

Table 3. The phenotypic results at 30 °C and 41 °C.

Isolate	Growth					
	30 °C			41 °C		
	YPDA Media	YPDA pH 3.5 Media	YPA-Molase 35°brix Media	YPDA Media	YPDA pH 3.5 Media	YPA-Molase 35 °brix Media
K1	+++	+++	+++	++	++	++
K2	+++	+++	+++	++	++	++
K3	+++	+++	+++	++	++	++
K4	+++	+++	+++	+++	++	++
K5	+++	+++	+++	+++	+++	+++
K6	+++	+++	+++	++	++	++
K7	+++	+++	+++	++	++	++
K8	+++	+++	+++	++	++	++
K8	+++	+++	+++	++	++	++
K9	+++	+++	+++	++	+	++
K10	+++	+++	+++	++	++	++
K11	+++	+++	+	+	+	+
K12	+++	+++	+++	++	++	++
K13	+++	+++	+++	++	++	++
K14	+++	+++	+++	++	++	++
K15	+++	+++	+++	++	+++	+++
K16	+++	+++	+++	++	++	++
K17	+++	+++	+++	++	++	++
K18	+++	+++	+++	++	++	++
K19	+++	+++	+++	+	++	+
K20	+++	+++	+++	+++	++	+++
X	++	+++	+++	+	+	+

Where (+++): extremely good, (++): very good, and (+): moderate.

Isolates K5 (*Candida tropicalis* strain L2), K15 (*Candida tropicalis* strain MYA-3404), and K20 (*Candida tropicalis* strain Y277) were considered to be the distinguished yeasts in Table 3. Based on the results, *Candida tropicalis* resisted a high temperature (40 °C and above) [23] and acidic pH, which indicated its potential for use in the biotechnological process. *C. tropicalis* isolates showed an ability to produce 6.55% (*v*/*v*) and 4.58% (*v*/*v*) ethanol at 30 °C and 42 °C [24]. It also resisted the presence of inhibitors such as furfural, which hinder cell growth and decrease bioethanol productivity [25]. Furthermore, *C. tropicalis* is also capable of releasing α-amylase for fermentation [26].

In conclusion, *Saccharomyces cerevisiae* is generally used in fermentation. On the other hand, the application of novel yeast from coconut water in the fermentation process is promising due to its high temperature tolerance, acidity, and sugar content, as these can lead to a quicker fermentation by reducing the cooling cost, inhibitors, and acidity treatment with lower production expenses. In addition, the phenotypic test can be designed based on the conditions that are needed in the production. It has a flexibility setting in terms of

regulating the temperature, pH, and molasses concentration. Isolates K5 (*Candida tropicalis* strain L2), K15 (*Candida tropicalis* strain MYA-3404), and K20 (*Candida tropicalis* strain Y277) have a high capacity to achieve this condition.

5. Conclusions

The three discovered isolates were K5 (*Candida tropicalis* strain L2), K15 (*Candida tropicalis* strain MYA-3404), and K20 (*Candida tropicalis* strain Y277), which were *Candida tropicalis* species from the isolation and identification of yeast from coconut water. These isolates probably contribute to fermenting molasses because they survived the phenotypic test with a high temperature, high acidity, and high molasses concentration. These novel yeasts have the potential to effectively and efficiently ferment molasses for further studies in bioethanol production.

Author Contributions: Conceptualization, G.K. and H.H.; methodology, G.K. and H.H; validation, H.H.; formal analysis, G.K.; investigation, G.K.; resources, G.K.; data curation, G.K.; writing—original draft preparation, G.K.; writing—review and editing, G.K., H.H., D.O., N.N. and P.L.H.; visualization, G.K.; supervision, H.H., N.N. and P.L.H. All authors have read and agreed to the published version of the manuscript.

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