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Diversity and potency of indigenous yeast from some palm juices for bioethanol production

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Abstract. Widyaningrum T, Suharjono S, Ardyati T, Aulanni'am A. 2020. Diversity and potency of indigenous yeast from some palm juices for bioethanol production. *Biodiversitas* 21: 318-325. Main energy source commonly used by the community comes from fossil energy, especially petroleum. The crisis of energy showed that Indonesia's fossil energy reserves are limited. Based on this fact it is important to develop alternative energy that environmentally friendly and sustain, especially bioethanol. The objective of this research was to diversity and potency of indigenous yeast from palm juice of *Arenga pinnata* Merr., *Cocos nucifera* L., *Nypa fruticans* Wurmb., and *Borassus flabellifer* L. for bioethanol production. Yeast from juice of the four species of palm was isolated using Yeast Malt Extract Agar (YMEA). Those isolates were screen base on ethanol production in coconut water media. The potential of yeast isolates was identified base on 18S DNA sequence similarity. The four potential isolates of yeast were A3A from *A. pinnata*, K1A from *C. nucifera*, N3E from *N. fruticans*, and S1A from *B. flabellifer* have highest production of ethanol are 8.6 %, 14.2 %, 9.0 %, and 9.2 % respectively. Four potential isolates of yeast were A3A and N3E, K1A, and S1A have relationship with *Saccharomyces cerevisiae* NRRL Y-12632^T, *Pichia manshurica* IFO 10726^T, and *Candida tropicalis* ATCC 20615 respectively.

Keywords: 18S DNA, bioethanol, indigenous yeast, palm juice

INTRODUCTION

Fossil fuels, especially petroleum, coal, and natural gas, are a major source of energy for most industries and are still the most important raw material for energy generation in the world. Currently, the world energy market value of about 1.5 trillion dollars is dominated by fossil fuels (Goldemberg 2006). However, these sources are no longer considered sustainable, and their availability decreases. (Shafiee and Topal 2009) predicted that oil, coal, and gas would only be sequentially left around 35, 107, and 37 years respectively. On the other hand, the use of fossil fuels as the main energy resources caused the arising of worldwide problems such as environmental pollution and global warming (Hoekman 2009; Kiran and Kumar 2014). There is inevitable depletion of the world's energy supply, there has been an increasing worldwide interest in alternative sources of energy (Ali et al. 2011). Bioethanol is one of the main renewable energy sources which is undoubtedly, a future fuel. Bioethanol has a higher octane number relative to that of gasoline alone, its use as a blender with gasoline reduces the emission of CO₂, NO_x, and hydrocarbons after combustion. The use of ethanol shows high compression ratio and increased energy production (4) a combustion engine (Balan et al. 2013). Production of fuel ethanol through fermentation appears to be a potential alternative to fossil fuel and can be used as an only fuel in vehicles with devoted apparatuses or in fuel

mixtures. Ethanol is presently derived from sugars, starches, and cellulosic materials. Unconventional cellulosic materials as seagrass and potato (21) our also produced ethanol (Rani et al. 2010; Basavaraj et al. 2013). Three main reasons for the production of bioethanol from cellulosic biomass are: (i) it is renewable, (ii) doesn't emit harsh gases like CO₂, SO₂, NO₂ into the environment, and (iii) it holds the key factor to the economy. The low-cost fermentation substrate that can meet the demands of oil of the future is lignocellulosic biomass. The cellulose biomass consists of mainly waste of inedible cellulose fibers that form the stems (39) and branches of most plants. Grain crops, switchgrass, crop residues like corn stalks, wheat straw, rice straw, grass dipping, and wood residues are the many forms of cellulosic biomass (22) arathiraja et al. 2014).

Nowadays bioethanol productions from cellulosic materials offer a solution to some of the recent environmental, economic, and energy problems facing worldwide (Kumar and Pushpa 2012). Bioethanol is generally produced by liquefaction and saccharification of starch using alfa-amylase and glucoamylase enzymes. The sugar slurry undergoes fermentation process by bacteria, yeast, or other fermenting microorganisms (Hanif et al. 2017). Various researches have been carried out for producing ethanol from several biomasses include micro and macroalgae (John et al. 2011), molasses (Wardani and Pertiwi (2013); Shamim et al. 2016), palm juice of *Cocos nucifera* (Saputra et al. 2012); palm juice of *Nypa* (Yenti

2013; Muhammad et al. 2016), Palm juice of *Borassus flabellifer* (Naknean et al. 2010) and *Sargassum* (Saputra et al. 2012; Borines et al. 2013; Widyaningrum et al. 2016) previous studies utilizing *Saccharomyces cerevisiae* to ferment of molasse and micro and macroalgae were produced ethanol 2.71-94.0 % (John et al. 2011; Riyanti 2011; Wardani and Pertiwi 2013).

Indonesia is one of tropical countries that have many palm plantations such as *Arenga pinnata* Merr., *Cocos nucifera* L., *Nypa fruticans* Wurmb., and *Borassus flabellifer* L. The most important product of palm is the sap or juice. The tapping process of palm sap involves the bruising of the interior of the developing inflorescences by means of a wooden mallet or tong, thereby stimulating sap flow. Sap is collected by cutting the end/tip of the inflorescence. Palm sap is rich in sugars (10-17%) (Naknean et al. 2010). The objective of this study was to diversity and potency of indigenous yeast from palm juice of *A. pinnata*, *C. nucifera*, *N. fruticans*, and *B. flabellifer* for bioethanol production.

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MATERIALS AND METHODS

Sampling of palm juice and isolation of yeast

Palm juice of *Arenga pinnata* Merr. and *Cocos nucifera* L. was taken from Samigaluh, District of Kulonprogo, Province of Yogyakarta, while *Nypa fruticans* Wurmb was taken from the District of Cilacap, and *Borassus flabellifer* L. was taken from District of Rembang, Province of Central Java, Indonesia. Each of palm juices was taken 250 mL (3 samples/replications) in a bottle and put in an icebox and then it was measured pH, reducing sugar content, and ethanol levels. Sample of each palm juice as much as 25 mL was diluted with 225 mL of physiological salt as 10^{-1} dilution. This sample suspensions were made series dilution until 10^{-9} . The samples suspension at each dilution was taken 0.1 mL inoculated into a sterile Petri dish and poured 15 mL of YMEA (Yeast Malt Extract Agar) medium. The culture was incubated at 25 °C for 48 hours and each type of yeast colony that grown was calculated.

Isolate of yeast was purified according to spread plate method. The colony of yeast was suspended into 10 mL of physiological salt and made series dilution until 10^{-6} . Suspension of yeast 0.1 mL was spread by Drigalsky glass rod on the surface of YMEA medium in Petri dish. The yeast culture was incubated at 25 °C for 48 hours (Aung et al. 2013). The pure culture of yeast was verified by Gram staining. In this research, the palm juice was characterized include pH, ethanol content, sugar content, The data were analyzed of variance with significance different ($\alpha=0.05$ using SPSS program version 16.0 The pure yeast stock then was screened to obtain the highest potential isolates on ethanol production.

Bioassay of yeast to produce ethanol

This experiment was carried out according to Completely Randomized Design with yeast isolate and incubation time as treatments. Parameters of the experiment that observed include reducing sugars, yeast

cell number, and ethanol concentration. One loop of yeast was inoculated into 100 mL of coconut water medium and it incubated at 30° C for 24 h. The suspension of yeast culture with similar cell density was taken 10 mL inoculated into 100 mL of coconut water (*C. nucifera*). The culture was fermented at room temperature for six days (Blanco et al. 2012). The parameters of fermentation culture include sugars by DNS method (Jackson and Jayanthi 2014), cell number of yeast, and ethanol concentration was measured at 0, 2, 4, and 6 day incubation time. The data was analyzed of variance with significance different $\alpha:5\%$ using SPSS program version 16. If treatment gave significant effect followed by Duncan Multiple Range Test (DMRT) to determined selected isolates that having highest potency to produce ethanol.

Optimization of selected yeast to produce ethanol

This experiment was carried out according to Completely Randomized Design with treatment consist of yeast isolate and incubation time. Parameters of fermentation that observed include reducing sugars, number of yeast cells, and ethanol concentration. The selected yeast culture was inoculated 1 loop into 100 mL of coconut water. The yeast culture was incubated at 30° C for 24 h. The suspension of yeast culture with similar cell density as much as 10 mL was inoculated into 100 mL coconut water media with varies of pH and reducing sugar concentration and it incubated six days (Blanco et al. 2012). The suspension of culture at 6 days fermentation, were measured of reducing sugars by DNS method (Jackson and Jayanthi 2014), cell number, and ethanol concentration. The data was analyzed of variance with $\alpha: 5\%$ using SPSS program version 16. If treatment gave significant effect followed by DMRT. Based on the DMRT test, the optimum for ethanol fermentation was determined.

Phylogenetic identification of yeast-based on 18S DNA

Extraction of yeast chromosomal DNA

DNA extraction was done based on (Elkins 2011). The yeast cells were grown in the liquid medium of YMEA. The yeast cells are harvested by centrifugation at 3,000 rpm for 15-30 minutes. The harvested cells were rinsed using 1 mL TE buffer and centrifuged 10,000 rpm for 15 minutes. The harvested cells were further broken down with 50 μ L lysozyme (50 μ g / mL) then shaken to homogeneous and incubated 37 °C for 30 min. To dissolve the membrane and enzyme proteins, GES reagent was added such as 250 μ L, homogenized until completely dissolved and incubated for 10 min at room temperature. Supplement plus 125 μ L ammonium acetate 7.5 M and placed on ice for 10 minutes. The separation of DNA from proteins and polysaccharides was done by adding 500 μ L chloroform to the solution, flipped 50 times, and centrifuged at 10,000 rpm for 10 min. Once completed centrifuges will form 3 layers and the DNA is at the bottom layer. The DNA deposits are taken using a blunt pipette and placed into a new Eppendorf. To form DNA threads, into the solution DNA isopropanol adds half the volume of the DNA solution, then flipped through the visible DNA threads, centrifuged at 10,000 rpm for 5 minutes until the DNA threads settle. Furthermore,

the precipitated DNA was washed with 70% cold ethanol, centrifuged again and the supernatant was discarded. The precipitated DNA was diluted for 10 min, dissolved in 100 μ L 0.2X TE buffer, and then the DNA concentration was measured using spectrophotometer at 260 nm wavelength.

The 18S DNA sequence amplification

Sequence amplification of 18S DNA with PCR

Sequences of 18S DNA were amplified using a general primer NS 1 (5'GTA GTC ATA TGC TTG TCTC 3') and NS 8 (5'TCC GCA GGT TCA CCT ACG GA3') (White et al. 1990). Amplification was performed on a 25 μ L reaction mixture containing 19 μ L sterile water, 25 green GoTaq (Promega), 2 μ L NS 1, 2 μ L NS 8, 2 μ L DNA. Amplicon was amplified under PCR conditions 94 °C for 3 min (initial denaturation), continued (94 °C, 1 min denaturation, 46 °C, 1-minute annealing, 72 °C, 1-minute elongation) 35 cycles and final extension at 72 °C, 5 minutes. The PCR product was then electrophoresed using 1% agarose gel (Herkert et al. 2015).

Sequencing and BLAST analysis of 18S DNA region

The amplicon of 18S DNA sequence was purified and it was sequenced using automatic sequencing machine ABI 3130 XL Genetic Analyzer using primer 18S DNA. The sequence was aligned with the reference sequence from GenBank of the National Center for Biotechnology Information (NCBI) to construct a phylogenetic tree based on the neighbor-joining algorithm with bootstrap 1000 replication using the MEGA 7.0 (<http://www.mega-software.net>) program (Kumar et al. 2018).

RESULTS AND DISCUSSION

Sampling of Palm juice and isolation of yeast

The sampling Palm juice of *A. pinnata*, *C. nucifera*, *N. fruticans*, *B. flabellifer*. found the initial condition of the palm juice produced was shown in Table 1. The initial palm juice, the lowest pH of the palm juice was obtained from *C. nucifera* L, the highest reducing sugar concentration was *B. flabellifer*., the highest ethanol concentration *C. nucifera* L, and the highest amount of yeast in *C. nucifera* The characteristic palm juices shown

that *C. nucifera* has the highest ethanol concentration and the highest amount of yeast, indicating that the yeast can form ethanol. The highest sugar concentration *B. flabellifer*. palm juice, but maybe sugar in *B. flabellifer*. palm juice can not be changed all into ethanol because ethanol in *B. flabellifer*. palm juice was lower than *C. nucifera* palm juice which has a lower sugar concentration than *B. flabellifer*. palm juice.

The results of isolation isolate of palm juices were obtained *A. pinnata* Merr found 5 isolates names A3B, A11E, A3A, A22A, A11B; *C. nucifera* found 5 isolates names K3D, K21A, K1C1, K2C, K1A; *N. fruticans* found 4 isolates names N3D, N3E, N1A, N3B, and *B. flabellifer*. palm juice found 4 isolates names S3D, S1A, S2D, S1C. The potential isolates are tested in producing ethanol with parameter days of fermentation (6th day), reducing sugar concentration, pH, and number of yeast.

Bioassay of yeast to produce ethanol

Table 1 shows that the highest ethanol concentration was obtained from palm juice of *C. nucifera* L, so each isolate of yeast was screened with palm juice of *C. nucifera* to produce ethanol. The potency of each isolate of yeast to produce ethanol with parameter days of fermentation (0,2,4, and 6 days), pH, reducing sugar concentration, and number of yeast. There were present in Figure 1.

Figure 1.A shows that during the fermentation process (6th day) there was a decrease in pH with the largest difference of up to 0.43 (in K3D isolate) although it was not significant, this was in accordance with the opinion of (Ogbona 2013) that a yeast, the pH range for growth can variously from 4 to 6. The environment that is too acidic or alkaline causes microorganisms is difficult to adapt. During the fermentation, the pH changes can be caused by fermentation results which are the acids or bases that produced during the growth of microorganisms and organic components in the medium (Rahmawati 2010). The tendency of the fermentation medium increasingly acidic was caused by the ammonia used by the yeast cells as the nitrogen source was converted to NH₄⁺. The NH₄⁺ molecule will merge into the cell as R-NH₃. In this process H⁺ was left in the medium, so the longer the fermentation time the lower the pH of the medium (Lin et al. 2012). Based on this research, it can be seen that *A. pinnata*, *C. nucifera*, *N. fruticans*, and *B. flabellifer*. contain indigenous yeast which able to produce bioethanol.

Table 1. The density of cell and biochemical characteristic of palm juices

Parameters	-	Types of palm juices			P. value	
		<i>A. pinnata</i> <i>B.</i>	<i>C. nucifera</i>	<i>N. fruticans</i>		<i>B. flabellifer</i>
Total Plate Count (CFU/g)		23.08 ± 0.97 a	56.75 ± 0.88 d	54.28 ± 0.98 c	38.2 ± 0.3 b	0.18
pH		5.61 ± 0.25 c	3.62 ± 0.11 a	4.28 ± 0.11 b	4.39 ± 0.07 b	0.99
Sugar reduction (mg/mL)		13.01 ± 0.88 a	17.09 ± 0.55 b	33.38 ± 0.99 c	43.35 ± 0.29 d	0.04
Etanol concentration (%)		2.89 ± 0.56 b	4.63 ± 0.19 c	2.01 ± 0.35 a	2.13 ± 0.08 a	0.78

Note: The same letter within each column do not differ significantly ($p > 0.05$) according to the Duncan test.

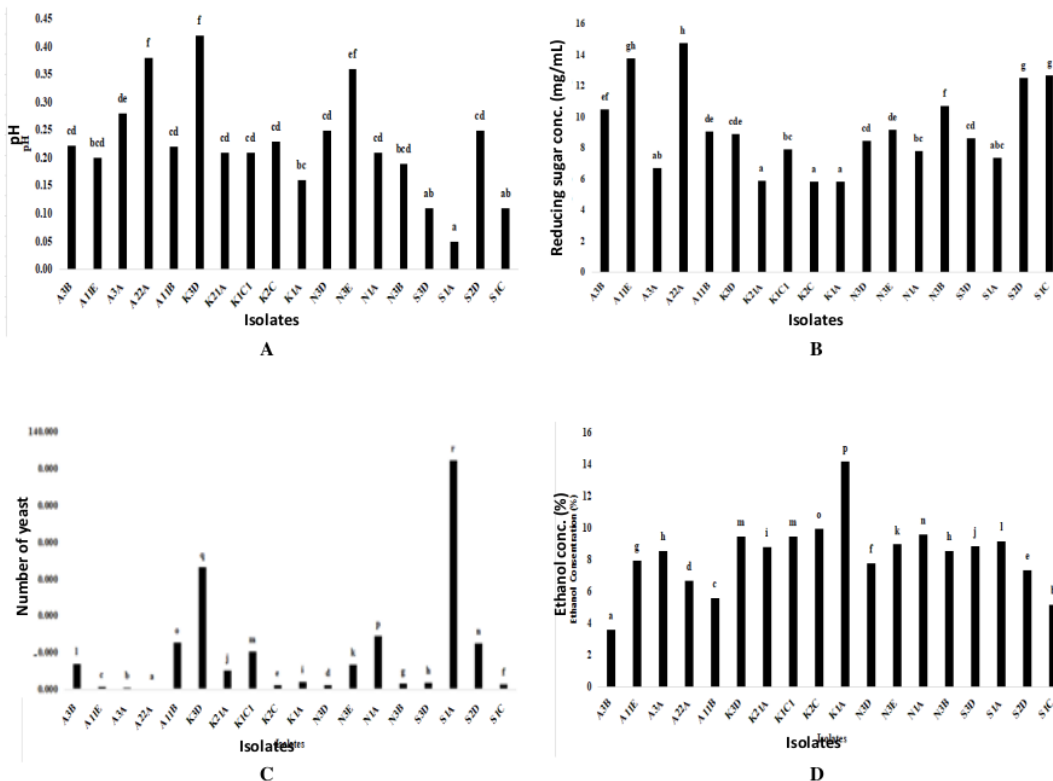


Figure 1. Potency of yeast isolates on ethanol production. A. The pH of isolates, B. Reducing sugar concentration, C. Number of yeast, D. Ethanol concentration. The same letter above each histogram show each parameter is not significantly different ($p > 0.05$) among isolates

The yeast isolates that having potency to produce ethanol on 6 days fermentation, with ten isolates of superior isolates (Figure 1.D), i.e. A3A, A11E isolates from *A. pinnata*, K1A, K2C, K1C1, isolates from *C. nucifera*, N1A, N3E, N3B, isolates from *N. fruticans*, S1A and S2D isolates from *B. flabellifer.*, with production of ethanol content of 8.6%, 8%, 14.2%, 10%, 9.5%, 9.6%, 9%, 8.6%, 9.2%, and 7.4%, respectively. The days of fermentation with the highest bioethanol fermentation was 6 days, because the days time it was entering an exponential phase where the number of microbes of yeast and enzymes was secreted at the optimum amount. The longer the fermentation process, the activity of yeast as microbes which become degrading agents of sugar into bioethanol was also decreasing (Shamim et al. 2016). The lag phase was the adjustment period and the time of 6 days was the optimum time, which was exponential or logarithmic, which that bioethanol as the primary metabolite was produced, whereas after more than 6 days yeast cells enter the stationary phase and death, so that the bioethanol produced decreases (Apriwida 2013).

Fermentation time affects the growth of microorganisms. Shorter fermentation time causes inefficient fermentation due to inadequate growth of microorganisms. On the other hand, longer fermentation time gives toxic effect on microbial growth especially in batch mode due to the high concentration of ethanol in the fermented broth. Complete fermentation can be achieved at lower temperature by using longer fermentation time which results in lowest ethanol yield. Agitation rate controls the permeability of nutrients from the fermentation broth to inside the cells and removal of ethanol from the cell to the fermentation broth. The greater the agitation rate, the higher the amount of ethanol produced. Besides, it increases the amount of sugar consumption and reduces the inhibition of ethanol on cells. The common agitation rate for fermentation by yeast cells is 150-200 rpm. Excess agitation rate is not suitable for both ethanol production as it causes limitations to the metabolic activities of the cells (Zabed et al. 2014).

Optimization of selected yeast to produce ethanol

The results of the screening shown that obtained ten superior isolates, i.e. A3A, A11E isolates from *A. pinnata*, K1C1, K1A, K2C isolates from *C. nucifera*, N3E, N3D, N1A isolates from *N. fruticans*, S1A and S2D isolates from *B. flabellifer*, so the next step these superior isolates were

tested for bioethanol produce using coconut water (assuming coconut water is part of the waste that still contains glucose) based on pH treatment, sugar addition, and temperature treatment, the results as shown on Figure 2.

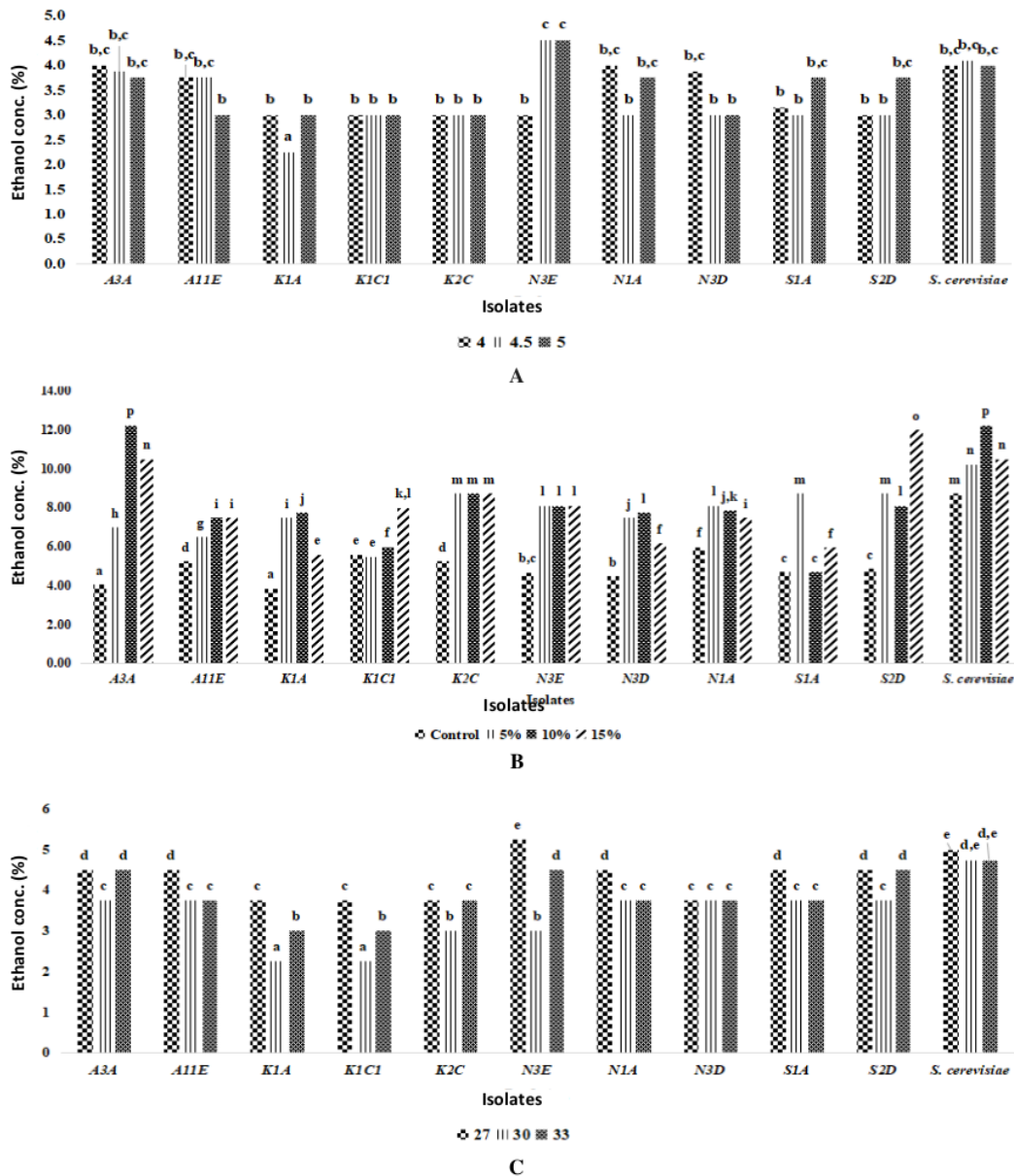


Figure 2. Ethanol concentration. A. pH treatment, B. Sugar concentration treatment, C. Temperature treatment. The same letter above each histogram show each parameter is not significantly different ($p > 0.05$) among isolates

The results with pH treatment (Figure 2.A) shown that isolate N3E was the highest bioethanol content on pH 4.5 and 5 (4.5%). According to Piriya et al. (2012), optimum ethanol could be obtained if the range of pH for the fermentation using *P. stipitis* was around 4.5-5.5. Meanwhile, Narendranath (2005) explained, optimum ethanol could be achieved if the pH range for fermentation by *S. cerevisiae* was around 5.0-5.5. The result with sugar addition treatment (Figure 2.B) shown that isolate A3A was the highest bioethanol content on 10% sugar addition (12.25%). The sugar concentration required for the optimum ethanol was 120 g/L (*P. stipitis*), but *S. cerevisiae* the sugar concentration was relatively low. The increase in sugar concentration up to a certain level caused fermentation rate to increase. However, the use of excessive sugar concentration will cause steady fermentation rate. This is because the concentration of sugar is beyond the uptake capacity of the microbial cells. Generally, the maximum rate of ethanol production is achieved when using sugars at a concentration of 150 g/L. The initial sugar concentration also has been considered as an important factor in ethanol production. High ethanol productivity and yield in batch fermentation can be obtained by using higher initial sugar concentration. However, it needs longer fermentation time and higher recovery costs (Zabed et al. 2014).

The result with temperature treatment (Figure 2.C) shown that isolate N3E was the highest bioethanol content on 27°C (5.25%). Temperature is one of the most important parameters in the production of ethanol since enzymatic hydrolysis and glucose fermentation rates depend upon the temperature. Generally, the fermentation temperature has a greater influence on the rate of fermentation. As the fermentation temperature increases the rate of growth as well as the rate of product formation increase. But there is a limitation for bioprocesses a higher temperature may not favor the growth, the cells may die, the enzymes may denature and the rate of product formation may be affected (Umamaheswari et al. 2010).

The growth rate of the microorganisms is directly affected by the temperature (Chanchai and Henschke 1999). High temperature which is unfavorable for cell growth becomes a stress factor for microorganisms (MarelneCot and Loret 2008). The ideal temperature range for fermentation is between 20 and 35°C. Free cells of *S. cerevisiae* have an optimum temperature near 30°C whereas immobilized cells have slightly higher optimum temperature due to its ability to transfer heat from particle surface to inside the cells (Liu and Shen 2008). Moreover, enzymes which regulate microbial activity and fermentation process are sensitive to high temperature

which can denature its tertiary structure and inactivates the enzymes (Phisalaphong and Srirattana 2010).

Phylogenetic identification of yeast-based on 18S DNA

The results of screening using coconut water shown that the most superior isolates producing bioethanol are A3A (*A. pinnata*), K1A (*C. nucifera*), N3E (*N. fruticans*), and S1A (*B. flabellifer*). Furthermore, to find out the name of the species from each isolate, it was identified using 18S DNA with the results as shown in Figure 3.

Based on identification of the 18S DNA, isolates A3A and N3E were seen similar to *Saccharomyces cerevisiae* NRRL Y-12632, this indicates that the yeast of the indigenous palm juice of *A. pinnata* and *N. fruticans* had the ability to convert glucose into bioethanol better than the other isolates. It was seen that isolates A3A and N3E with pH treatment (Figure 2.A) shown the highest levels of bioethanol (4.5%). In the treatment of adding 10% sugar (Figure 2.B) the A3A isolates also shown the highest level of bioethanol (12.25%) same as control. At the temperature treatment (Figure 2.C) N3E isolate shown the highest ethanol content (5.25%). According Ye et al. (2016) *Saccharomyces cerevisiae* is a key microorganism that could produce bioethanol. *Saccharomyces cerevisiae* a well-established organism for bioethanol production (Mannan et al. 2018). *S. cerevisiae* is the most commonly employed yeast in industrial ethanol production as it tolerates a wide range of pH (et al. 2012). According to Mussato et al. (2012) certain yeast strains such as *Pichia stipitis* (NRRL-Y-7124), *S. cerevisiae* (RL-11) and *Kluyveromyces fragilis* (Kf1) were reported as good ethanol producers from different types of sugars.

The results of the identification classified as valid because the index similarity was more than 95%. *Candida tropicalis* is also found in palm juice *B. flabellifer* from Thailand parallel with *Kloeckera apiculata*, *Kloeckera japonica*, *Candida krusei*, and *Candida valida* (Tuntiwongwanich and Nanon 2009). Palm juice of *Borassus* is an academic from Burkina Faso, West Africa there is also yeast *Candida tropicalis* parallel with some other yeast-like *Saccharomyces cerevisiae* and *Candida pararugosa* (Ouoba et al. 2012).

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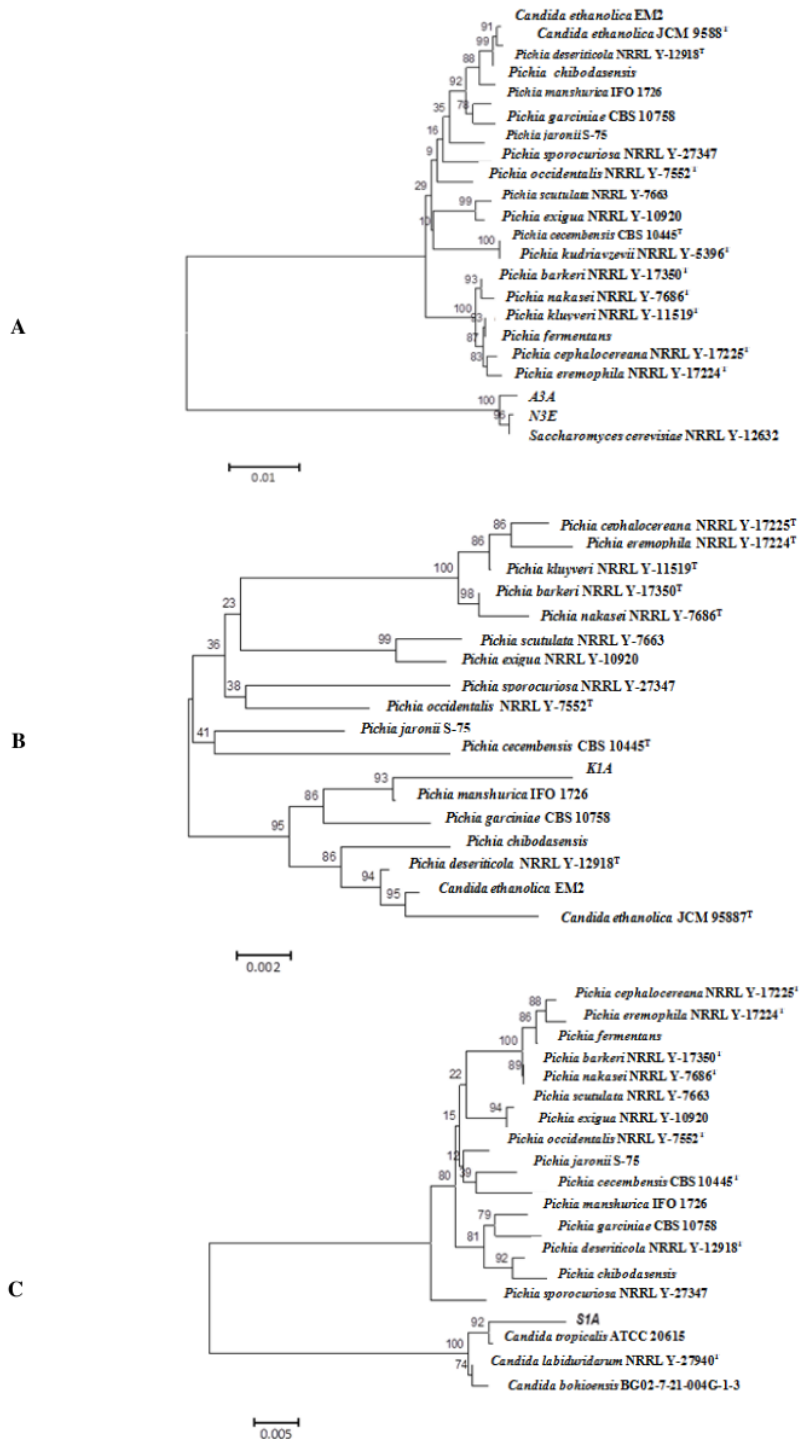


Figure 3. Phylogenetic tree. A. Isolates A3A and N3E, B. Isolate K1A, C. Isolate S1A. The reference strain based on the 18S DNA sequence using the neighbor-joining Tamura-Nei algorithm with bootstrap 1000 replication

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