

Exploration, screening and identification of indigenous yeast from some palm juices for bioethanol production

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Abstract. Widyaningrum T, Febrianti N, Prastowo I, Saifuddin MF, Permadi A. 2022. Exploration, screening and identification of indigenous yeast from some palm juices for bioethanol production. *Biodiversitas* 23: 3984-3990. The major energy sources usually employed are originated mainly from fossil which can run out. Therefore, the creation of ecologically beneficial and long-lasting forms of alternative energy sources, such as bioethanol, is an absolutely necessary. This study aimed to explore, screen and to identify indigenous yeasts from some palm juices for bioethanol production. The isolates were screened in coconut water media for the degree of bioethanol production, the content of reducing sugar based on a DNS method, and the cell number based on optical density (600 nm). Furthermore, identification was performed using ITS and candidates of the highest bioethanol-producing ability including A3A, A11E from *Arenga pinnata*, K1C1, K1A, K2C from *Cocos nucifera* L., N3E, N3D, N1A from *Nypa fruticans*, S1A, and S2D from *B. flabellifer* L. were obtained. The respective bioethanol contents were 13.4%, 12.8%, 13%, 16%, 14.2%, 13.2%, 12%, 12.6%, 14.4%, and 13.35%. The results showed that the isolates of A3A and N3E were similar to those of *Pichia deserticola* CBS 7119^T. Meanwhile, the isolates of K1A and S1A were similar to that of *Pichia manshurica* CBS 240^T and *Candida tropicalis* ZA 021^T.

Keywords: *Arenga pinnata*, bioethanol, *Borassus flabellifer*, *Cocos nucifera*, indigenous, *Nypa fruticans*

INTRODUCTION

One of the current problems faced by Indonesia is energy consumption which is increasing and tends to be wasteful, while fossil energy reserves are running low (Faizah and Husaeni 2018). However, the exploration of energy resources is more focused on fossil energy, a non-renewable resource (Diputra and Baek 2018). The availability of these sources is decreasing because it is not sustainable (Shafiee and Topal 2009). It is estimated that oil, coal, and gas will only be available for 35, 107, and 37 years, respectively. Fossil fuels as the primary energy source have led to increased global problems, such as environmental pollution and global warming (Kiran et al. 2014). Meanwhile, Due to the inevitable depletion of the world's energy resources, interest in alternate sources has increased globally (Ali et al. 2011). Sources of bioethanol are one of the leading renewable energy sources and are undoubtedly the fuel of the future (Kusmiyati 2010; Purwoko et al. 2017). It has a higher octane number than gasoline and reduces CO₂, NO_x, and hydrocarbon emissions after combustion. Ethanol shows a high compression ratio and increased energy production in combustion engines (Balan et al. 2013).

The creation of fuel ethanol through fermentation appears to be a potential alternative to fossil fuel. It is a singular fuel source for vehicles with devoted apparatuses or a component of fuel mixtures. Materials containing starch, sugar and cellulose can be used as raw materials for

ethanol. Seagrass and potato flour contain ethanol (Rani et al. 2010; Ravikumar et al. 2011). The production of bioethanol has advantages: i) it is renewable; (ii) it does not emit harmful gases such as CO₂, SO₂, and NO₂, into the environment; and (iii) it holds the key economic factor. In the future, lignocellulosic biomass is the only low-cost fermentation substrate that might supply the present needs for oil. This cellulose biomass mainly consists of the waste of inedible cellulose, for instance, fibers that form the stems and branches of most plants. Furthermore, cellulose biomass can be found in grain crops, switchgrass, and crop residues such as corn stalks, rice straw, wheat straw, dipping grass, and wood (Bharathiraja et al. 2014).

Bioethanol production from cellulosic materials offers a solution to some of the recent environmental, economic, and energy problems (Srivastava et al. 2015). It is produced from starch rich crops by liquefaction and saccharification of starch using alfa-amylase and glucoamylase enzymes. The sugar slurry undergoes a fermentation process by bacteria, yeast, or other fermenting microorganisms (Hanif et al. 2017). Various research has been performed regarding the production of ethanol from several micro-and macro-algae (John et al. 2011); molasses (Shamim et al. 2016); (Wardani and Pertiwi 2013); palm juice of *Cocos nucifera* (Wijaya and Arthawan 2012); palm juice of *Nypa* (Chairul and Yenti 2013; Hadi et al. 2013); palm juice of *Borassus flabellifer* (Naknean et al. 2013); and *Sargassum* (Saputra et al. 2012; Borines and Cuello 2013; Widyaningrum et al. 2016). Previous studies on molasses

and micro-and macro-algae using *Saccharomyces cerevisiae* had an ethanol content ranging from 2.709%-94% (John et al. 2011; Riyanti et al. 2011; Wardani and Pertiwi 2013).

Indonesia is one of the tropical countries with many palm forests, such as *Arenga pinnata* Merr., *Cocos nucifera* L., *Nypa fruticans* Wurmb., and *Borassus flabellifer* L. The prime product of the palmyra palm is sap or juice. In order to get palm sap, a wooden mallet or tong is used to bruise the inside of the aborning inflorescences and thus induce sap flow. The sap contains about 10 to 17% sugar and it is collected by cutting the outer end at the head of the inflorescences (Naknean et al. 2010). This study explored, screened, and identified indigenous yeasts from the palm juices of *A. pinnata*, *C. nucifera*, *N. fruticans*, and *B. flabellifer* for bioethanol production.

MATERIALS AND METHODS

Sampling of palm juice and isolation of yeast

The palm juices of *A. pinnata* and *C. nucifera* were obtained from Samigaluh Kulonprogo, Yogyakarta, Indonesia. The juices of *N. fruticans* were taken from Cilacap, while *B. flabellifer* from Rembang, Central Java, Indonesia. A total of 250 mL in each sample was poured into a bottle and placed in a cooler to avoid fermentation. Samples of each palm juice (25 mL plus 225 mL of physiological salt (10^{-1})) were subjected to a series of dilutions of 10^{-9} . Furthermore, the sample suspensions were taken at 0.1 mL, inserted into a sterile petri dish, poured (15 mL of YMEA medium), and homogenized. The culture was incubated at 25°C for 48 h, and the yeast colony grown was calculated and then purified.

Each yeast colony was purified by the spread plate method. The units were calculated at 10 mL of physiological salt, and then serial dilutions to 10^{-6} was performed. Additionally, the suspensions were taken to 0.1 mL and then spread with Drigalski glass rods on the surface of the medium to make YMEA in the petri dish. Yeast culture incubation was carried out at 25°C for 48 h (Aung et al. 2013). The characterization of the samples was carried out by studying their the pH, ethanol content, sugar content, and total plate count. Subsequently, the data so obtained were analyzed using SPSS program version 16, and a p-value < 0.05 was considered statistically significant. The pure yeast stock was filtered to obtain the highest potential isolates according to ethanol production.

Screening yeast ethanol producers to get superior isolates

This study used a completely randomized design (RAL) for the yeast isolate and incubation time. The experimental parameters were reducing sugars, yeast cell counts, and ethanol content. The action step began with one loop of yeast colony 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 obtained were tabulated and analyzed at an α of 5% with the SPSS program version 16. The selected isolates were obtained according to the DMRT test.

Testing superior isolates using coconut water media

This study used a completely randomized design (RAL) to evaluate yeast cell counts, sugar content, and ethanol content. 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 α : 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.

Identification of yeasts based on ITS sequences

Chromosomal yeast DNA extraction

DNA extraction was performed based on the methods of Kalbande et al. (2012) and the yeast cells were grown using YMEA liquid media. Subsequently, they were harvested by centrifugation at 3,000 rpm for 15-30 m. The harvested cells were rinsed using 1 mL TE buffer and centrifuged at 10,000 rpm for 15 min. Further splitting was conducted with 50 L of lysozyme (50 g/mL) and then shaken to homogeneity before being incubated at 37°C for 30 min. The addition of GES reagent at levels up to 250 L was used to dissolve membranes and protein enzymes. The samples were then homogenized until fully dissolved and incubated at room temperature for 10 minutes. Additionally, supplement plus 125 μ L ammonium acetate 7.5 M was added, and the samples were placed on ice for 10 minutes. The separation of DNA from proteins and polysaccharides was achieved by adding 500 μ L chloroform to the solution, and flipped 50 times before centrifuging at 10,000 rpm for 10 min. The centrifugation formed 3 layers, and the DNA was in the bottom layer. The DNA deposits were taken using a blunt pipette and placed into a new Eppendorf tube, and to form the threads, an isopropanol was added at half the volume of the solution. It was flipped through the visible DNA threads and centrifuged for 5 minutes at 10,000 rpm. Furthermore, the precipitated DNA was washed using 70% cold ethanol, centrifuged again, and the supernatant was discarded. The precipitated DNA was diluted for 10 min and dissolved in 100 μ L 0.2X TE buffer, and the concentration was then measured using a spectrophotometer at a wavelength of 260 nm.

Sequence amplification of the ITS with PCR

The sequences of the ITS were amplified using primer ITS 1 (5'TCC GTA GGT GAA CCT TGC GG 3') and ITS 4 (5'TCC TCC GCT TAT TGA TAT GC 3') (Herkert et al. 2015). Amplification was performed on a 25- μ L reaction mixture containing 19 μ L sterile water, 25 green GoTaq (Promega), 2 μ L ITS 1, 2 μ L ITS 4, and 2 μ L DNA. Meanwhile, the amplicon was amplified with an initial denaturation for 3 min at 94°C, continued (94°C, 1 min denaturation, 50°C), 1 min annealing, 72°C, 1-minute elongation), 35 cycles, and a final extension for 5 minutes 72°C. The PCR product was then electrophoresed using 1% agarose gel (Mulyatni et al. 2011).

Sequencing and BLAST analysis

The purified PCR product was sequenced using the automatic sequencing machine ABI 3130 XL Genetic Analyzer used primer ITS 1/ ITS 4, and the result was analyzed with a BLAST at (<http://www.ncbi.nlm.nih.gov>) to identify the closely related species of *Saccharomyces* (Kumar et al. 2018).

RESULTS AND DISCUSSION

Sampling of palm juices and isolation of yeast

The sugar-containing sap from *A. pinnata*, *Borassus*, *Nypa*, and *Coconut* palms in Indonesia yields that can be tapped continuously from the trees' inflorescences. Furthermore, they are suitable for fermented ethanol production (Kismurtono 2012). The juices were biochemically tested, hence reducing sugar levels based on the DNS method, bioethanol levels based on an alcohol meter, calculated TPC, and measured pH were obtained, as shown in Table 1.

As shown in Table 1, the highest cell density and bioethanol levels occurred in the palm juice of *C. nucifera*. Isolation of yeast from four palm juices was conducted according to Aung et al. (2013) and resulted in 18 isolates, which were then screened.

Screening a yeast ethanol producer to get superior isolates

The highest level of bioethanol was obtained from the palm juice of *C. nucifera* (Table 1). Subsequently, there was a screening to acquire a productive isolate for producing bioethanol using palm juice of *C. nucifera*. After the statistical test (Duncan test <0.05), the treatment duration of fermentation that produced the highest content was the fermentation time of six days, which produced ten isolates of superior quality. These consist of A3A, A11E

isolates from *A. pinnata*, K1C1, K1A, K2C from *C. nucifera*, N3E, N3D, N1A from *N. fruticans*, S1A, and S2D from *B. flabellifer* with ethanol contents of 13.4%, 12.8%, 13%, 16%, 14.2%, 13.2%, 12%, 12.6%, 14.4%, and 13.35%, respectively.

The day with the highest bioethanol fermentation was six days because of an exponential phase where the microbes of yeast and enzymes were secreted at the optimum amount. The longer process reduces yeast activity as a degrading sugar agent into bioethanol (Shamim et al. 2016). There was an adjustment period of six days during which bioethanol was generated at its main exponential or logarithmic stage. After more than six days, the yeast cells entered the stationary phase and death, thus, the bioethanol produced decreased (Apriwida 2013).

Shorter times cause inefficient fermentation due to the inadequate growth of microorganisms. In contrast, longer times produce a toxic effect on microbial growth, especially in the batch mode, due to the high ethanol concentration in the fermented broth. Complete fermentation can be achieved at lower temperatures and longer times, resulting in the lowest ethanol yields. The agitation rate controls the permeability of the nutrients from the fermentation broth and the removal of ethanol from the cell. The greater the agitation rate, the higher the amount of ethanol produced.

Additionally, it increases the amount of sugar consumption and reduces the inhibition of ethanol in the cells. The standard agitation rate for fermentation by yeast cells is 150-200 rpm. Excessive rate is not suitable for smooth ethanol production because it causes limitation of the metabolic activities of the cells (Zabed et al. 2014).

The results showed that after the fermentation process, the pH conditions decreased. On the 6th day of fermentation, there was a decrease in pH, although this change was not significant, and it is in agreement with (Ogbonda et al. 2013). For yeast, the growth pH range can vary from 4 to 6, and highly acidic or alkaline environment is challenging for the adaptation of microorganisms. During the process, changes in pH can be caused by fermentation products, namely acids or bases. This can result to change during the growth of microorganisms and organic components in the medium (Rahmawati 2010). The tendency of the fermentation medium to be increasingly acidic was caused by the ammonia used by the yeast cells as the nitrogen source was converted to NH_4^+ . Furthermore, the molecule will merge into the cell as R-NH_3 , while H^+ is left in the medium, therefore, longer fermentation times mean lower pH values (Lin et al. 2012). Based on this research, *A. pinnata*, *C. nucifera*, *N. fruticans*, and *B. flabellifer* contain indigenous yeast capable of producing bioethanol.

Table 1. The density of the cell and biochemical characteristics of palm juices

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

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

Test of superior isolates using coconut water media

The screening showed that A3A and A11E isolates from *A. pinnata*, K1C1, K1A, K2C from *C. nucifera*, N3E, N3D, N1A from *N. fruticans*, S1A and S2D from *B. flabellifer* were superior. Therefore, they were tested for bioethanol production using coconut water based on pH and temperature treatments, as well as sugar addition, as shown in Figure. 1, 2, and 3.

The isolate N3E has the highest bioethanol content of 4.5% at pH 4.5 and 5 (Figure 1.) Isolate A3A has the highest bioethanol content at 12.25% (Figure 2) To achieve the highest level of bioethanol, a condition of pH 4.5 and 5 should be applied, and it should contain 10% of sugar. According to Dash et al. (2015), an optimum ethanol concentration could be obtained when the pH range for the fermentation using *P. stipitis* is approximately 4.5-5.5. Subsequently, Ogbonda and Kiin-Kabar (2013) explained

that the pH range for fermentation using *Saccharomyces cerevisiae* to achieve optimum ethanol is around 5.0-5.5. The sugar concentration required for the optimum ethanol is 120 g/L for *P. stipitis*, while for *Saccharomyces cerevisiae*, it is relatively low.

Sugar concentration is directly proportional to the fermentation rate. However, excessive concentration cause a steady fermentation rate. This is because the sugar level is beyond the microbial cells' uptake capacity. The maximum ethanol production rate is achieved when using a concentration of 150 g/L. The initial sugar concentration has also been considered an essential factor in ethanol production. High productivity and batch fermentation yield can be obtained using increased initial sugar concentrations. However, this requires longer fermentation times and higher recovery costs (Zabed et al. 2014).

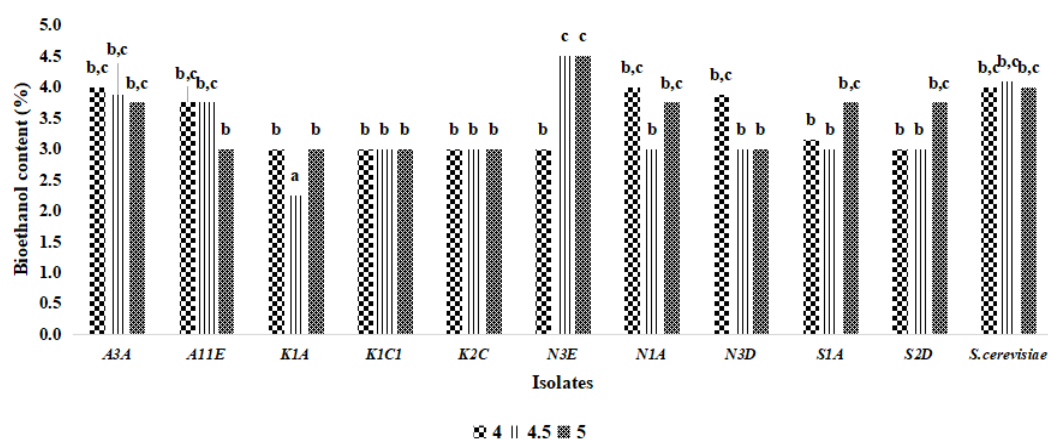


Figure 1 Bioethanol contents from superior isolates with pH treatment (4, 4.5, and 5)

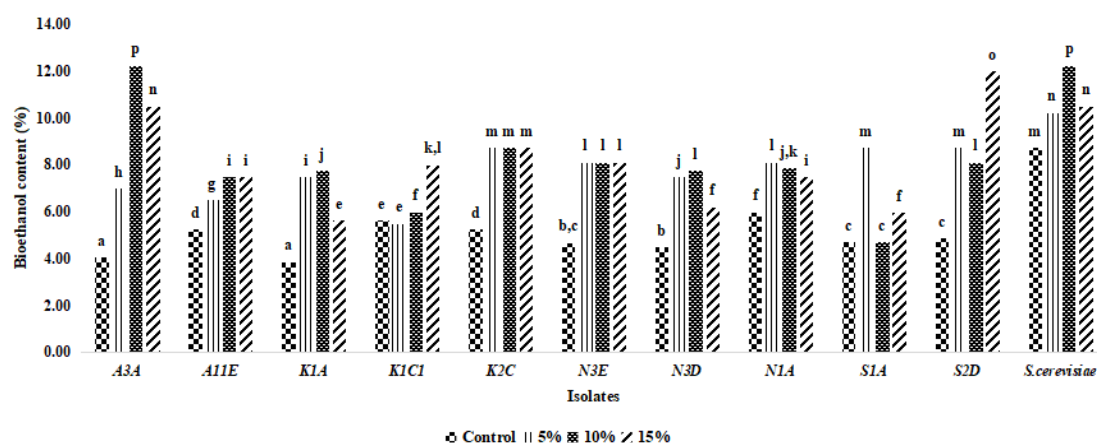


Figure 2. Bioethanol content from the superior isolates with the concentrations of sugar treatment (control, 5%, 10%, and 15%)

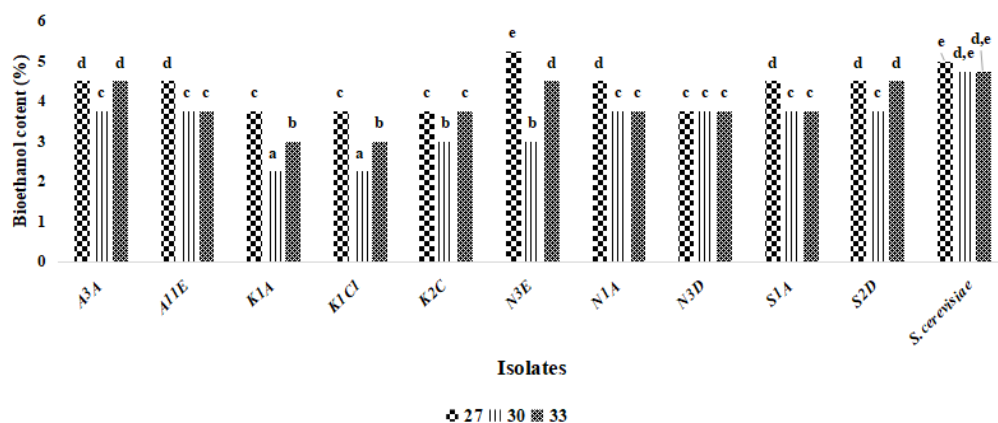


Figure 3. Bioethanol contents of the superior isolates with temperature treatment (27°C, 30°C, and 33°C)

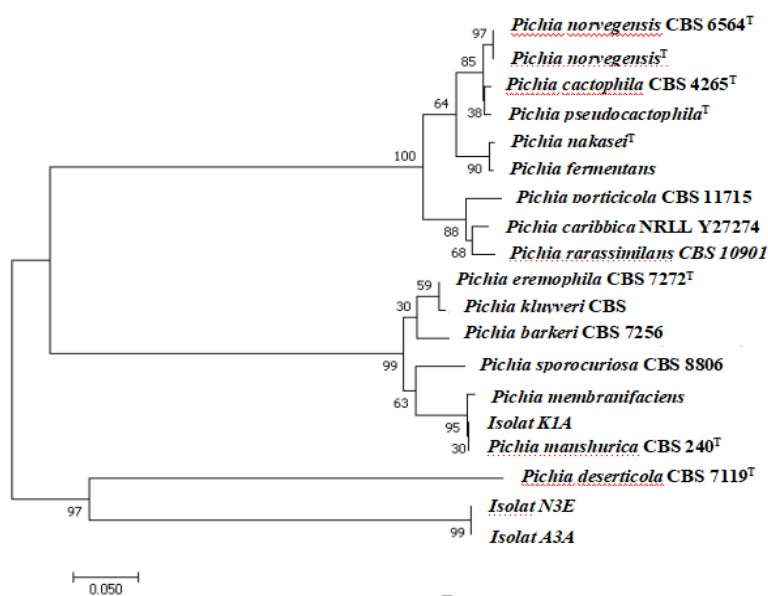


Figure 4. Phylogeny tree of the K1A, N3E, and A3A isolates that references *Pichia* based on a neighbor-joining algorithm

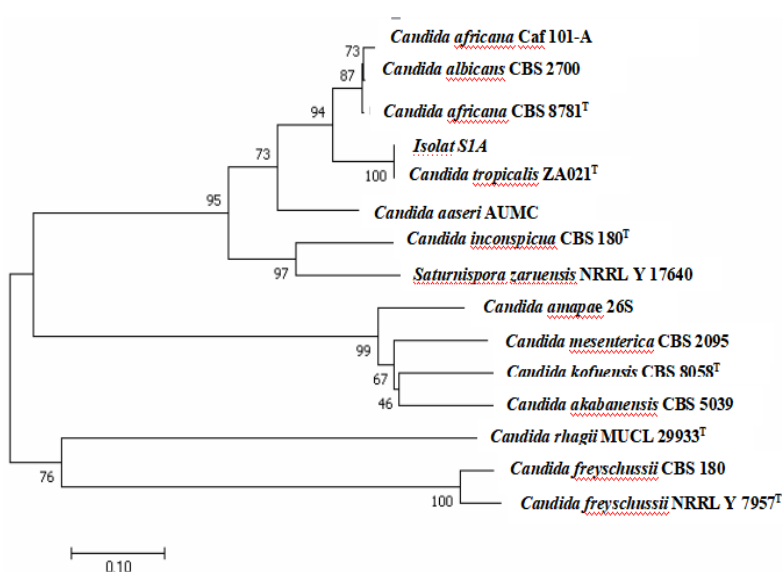


Figure 5. Phylogeny tree of the S1A isolate and references *Candida tropicalis* ZA 021^T based on Neighbor-joining algorithm

The temperature of the isolate N3E was the highest, with 5.25% bioethanol content at 27°C. It is critical in ethanol production because enzymatic hydrolysis and glucose fermentation rates depend on this parameter. The fermentation temperature increases with the growth and product formation rate. However, there is a limitation for bioprocesses, for instance a higher temperature may not favor the growth. The cells, enzymes, and the rate of product formation may be affected (Umamaheswari et al. 2010).

The temperature directly affects the growth rate of microorganisms (Mohd Azhar et al. 2017). High temperature, unfavorable for cell growth, becomes a stress factor for microorganisms (Tronchoni et al. 2009). The ideal range is between 20°C, 35°C, and free cells of *Saccharomyces cerevisiae* have an optimum temperature near 30°C. Meanwhile, immobilized cells have slightly higher optimum temperatures due to their abilities to transfer heat from their particle surfaces to the insides of the cells (Phisalaphong and Srirattana 2010). Enzymes that regulate microbial activity and fermentation processes are sensitive to high temperatures, denature their tertiary structures, and inactivate the enzymes (Wan et al. 2012).

The most superior isolates producing bioethanol are A3A (*A. pinnata*), K1A (*C. nucifera*), N3E (*N. fruticans*), and S1A (*B. flabellifer* L). Furthermore, the identification was made using ITS 1 and 4 to obtain the name of the species from each isolate (Figures 4 and 5). Based on the phylogeny tree shown in Figure 4, the K1A isolate is similar to *Pichia membranifaciens*, with an index of 95%. Meanwhile, N3E and A3A are similar to *Pichia deserticola* CBS 119T with an index similarity of 97%. The S1A is similar to *Candida tropicalis* ZA 021^T with an index of 100% (Figure 5). The results of the identification and classification are valid because the similarity index is more than 95%. Furthermore, *Candida tropicalis*, *Kloeckera apiculata*, *Kloeckera japonica*, *Candida krusei*, and *Candida valida* are found in the palm juice of *B. flabellifer* from Thailand (Tuntiwongwanich and Leenanon 2009). The palm juice of *Borassus* is an academic lend from Burkina Faso, West Africa, and there is also the yeast of *Candida tropicalis*, similar to *Saccharomyces cerevisiae* and *Candida pararugosa* (Ouoba et al. 2012).

In conclusion, the superior isolate from each source of palm juice obtained isolates A3A (*A. pinnata*), K1A (*C. nucifera*), N3E (*N. fruticans*), and S1A (*B. flabellifer* L). The molecular identification showed that the A3A and N3E isolates, having ethanol contents of 13.4 % and 13.2 %, are similar to *Pichia deserticola* CBS 119T with an index of 97%. The K1A and S1A isolates are similar to *Pichia membranifaciens* and *Candida tropicalis* ZA 021^T with an index of 95% and 100%, as well as ethanol contents of 16% and 14.4%.

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