ASM Sc. J., 16, Special Issue 1, 2021 for SCIEMATHIC2019, 166-171

Identification indigenous Yeast from Palm Juice *Cocos nucifera* L for Bioethanol Production

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The search for alternative and renewable fuel sources has become a major concern all over the world because of its environmental friendliness and environmental sustainability. Sources of raw materials include bioethanol. Bioethanol as raw material to replace fossil fuels because fossil fuels cause global warming and the greenhouse effect. High carbon source material is a source of bioethanol that renewable, effective, and abundant. Bioethanol from palm juice can be a promising technology because of its high sugar content. The goals of this research is to screen and identify indegenous yeast from palm juice *Cocos nucifera* L which is potential for bioethanol production. Isolation of yeast from palm juice *C.nucifera* L using YMEA medium. Purification used coconut water media. Screening used several parameters: bioethanol content (using alcohol meter), reducing sugar content (DNS method), and cells number (spectrophotometer 600 nm). Identification used 18S DNA. Five yeast isolates were produced, namely K3D, K21A, K1C1, K2C, and K1A. The superior isolates was K1A (16%). The isolate was similar with *Pichia manshurica* strain IFO 10726 with 99.99 similarty value.

Keywords: *Cocos nucifera*; indigenous; bioethanol; *Pichia manshurica* strain IFO 10726

I. INTRODUCTION

Fossil fuels, especially petroleum, natural gas and coal are the main concerns throughout the world. This fuel causes environmental impacts such as global warming and impacts on the greenhouse effect (Martins *et al*., 2019). Therefore needed the renewable, sustainable, and environmentally friendly, such as bioethanol. Bioethanol is one of the main renewable energy source that is environmentally friendly, the fuel of the future. Bioethanol has a higher octane number and is relatively safe to reduce CO2, NO2, and hydrocarbon emissions after a gasoline combustion engine (Balan *et al*., 2013).

Research on bioethanol production through fermentation has been widely published abroad using various strains of microorganisms, such as bacteria, yeasts, and fungi with different carbon sources (Stephanopoulos, 2007; Riyanti, 2011; Azhar *et. al*., 2017; Soleimani, Adiguzel & Nadaroglu, 2017) Generally bioethanol is produced with the favour of yeasts used type microorganisms with simple sugar carbon sources from molasses, sugarcane, or corn (Riyanti & Roger, 2009). Ingredient other than molasses, another ingredient that can be source of bioethanol is palm juice. Palm juice is containing sugar at concentration of 7.5% to 20% contained in the flowers of coconut, sugar palm, and palm trees (Dyanti, 2002). Palm juice contains water 87.66%, sugar

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12.04%, protein 0.36%, fat and ash 0.36% and 0.21%, respectively (Kurniawan *et al*., 2018).

Various studies have been carried out for producing bioethanol from several biomasses including micro and macroalgae (John *et al*., 2011); molasses (Sebayang, 2006); Wardani & Pertiwi (2013); coconut palm juice (Wijaya & Arthawan, 2012); palm juice of Aren (Ariyani & Chairul, 2015), nypha palm juice (Chairul & Silvia 2013; Hadi *et al*., 2013); and *Sargassum (*Borines & Cuello*,* 2013*;* Saputra *et. al.,* 2012*;* Widyaningrum *et al.,* 2016*)*. The purpose of this research was to screen and identify indigenous yeast from palm juice *Cocos nucifera L* which is potential for bioethanol production.

II. MATERIALS AND METHODS

A. Isolation of yeast from Palm juice Cocos nucifera L

Palm juice *Cocos nucifera L* was taken from Samigaluh, District of Kulonprogo, Province of Yogyakarta Indonesia. Isolate of yeast was purified according 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, Watanabe & Hashinaga, 2013). The pure culture of yeast was verified by Gram staining. In this research, the palm juice was characterised include pH, ethanol content, sugar content. The data were analysed of variance with significance different (α=0.05 using SPSS program version 16.0. The pure yeast stock then was screened to obtain the highest potential isolates on ethanol production.

B. Bioassay of Yeast to Produce of Ethanol

The study used a Completely Randomized Design with yeast isolate treatment, and incubation time. Parameters observed included reducing sugar by DNS method (Jackson & Jayanthy, 2014), number of yeast cells by Optical Density (OD) method, and ethanol content by Alcohol meter method. The step begins with taking 1 ose of pure isolate grown in 100 mL of coconut sap medium, incubated for 24 hours, making serial dilutions up to 10^{-9} OD size, counting the number of cells that grow, then making standard curves and regression equations. The 25 mL yeast culture was mixed with 225 mL of coconut sap, each incubated for 0, 2, 4, and 6 days, then measured the pH, measured the OD, and the OD values were equated, the number of cells, sugar, and ethanol content. Each treatment was repeated 3 times (Blanco *et al*., 2012). The data obtained were tabulated and analysed for variance: 5% using the SPSS version 16 program. If the treatment had a significant effect on real DMRT. Based on the DMRT test, selected isolates were obtained.

C. Phylogenetic Identification of Yeast base on 18S DNA Chromosomal yeasts DNA extraction

DNA extraction was done based on (Elkins 2013). DNA extraction was used iNtron Biotechnology Kit. The DNA concentration were measured using spectrophotometer at 260 nm wavelength.

D. Sequence amplification of 18 S DNA with PCR

The 18S rDNA sequences were amplified using the common primers NS 1 (5'GTA GTC ATA TGC TTG TCTC 3') and NS 8 (5'TCC GCA GGT TCA CCT ACG GA3') (Hadziavdic *et al*., 2014). Amplification was carried out in a 50 L reaction mixture containing 19 L aquadest, 25 green Go Taq (Promega), 2 µL NS 1, µL NS 8, 2µL DNA with DNA concentration of each isolate K3D, K21A, K1C1, K2C, K1A. Amplicons were amplified under PCR conditions at 94 °C for 3 min (initial denaturation), continued (94 °C, 1 min denaturation, 50 °C, annealing 1 min, 72 °C, 1 min extension) 35 cycles and final extension at 72 ° C, 5 minutes. The PCR product was then electrophoresed using 1% agarose gel (Herkert *et al*., 2015).

E. Sequencing and BLAST Analysis

The 18S rDNA sequence PCR amplicon was sent to First BASE Laboratories, Malaysia for sequencing. The sequencing results were aligned with the GenBank reference sequence from the National Center for Biotechnology Information (NCBI) to create a phylogenetic tree based on a neighbour-joining algorithm with a bootstrap 1000 replications, using the MEGA 7.0 program (http://www. Mega software.net) (Kumar *et al*., 2018).

III. RESULT AND DISCUSSIONS

The sampling Palm juice of *Cocos nucifera* L found the initial condition of the palm juice produced was shown on Table 1.

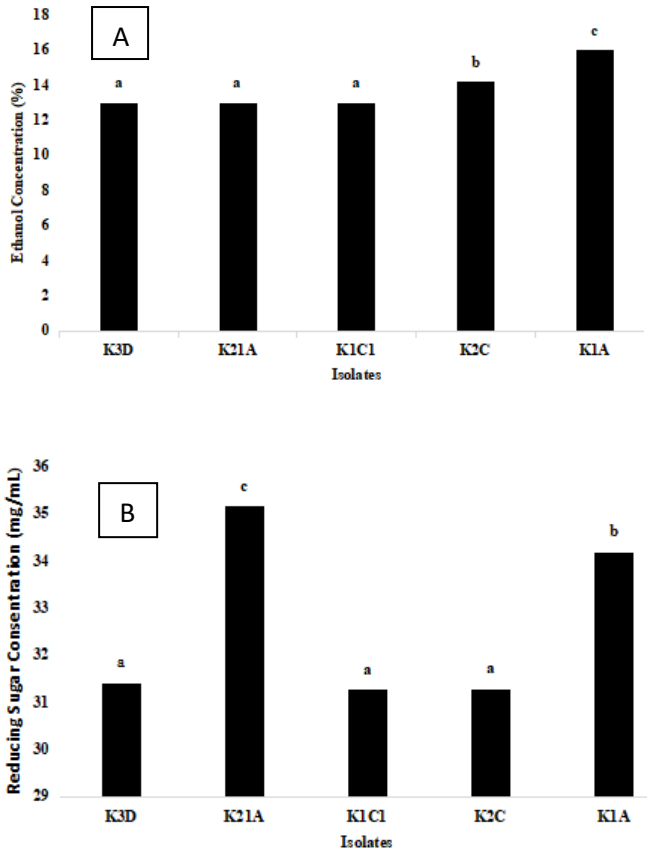
Table 1. The condition of palm juice *Cocos nucifera* L.

	pH	Reduction of Sugar (mg/mL)	Concentration of Ethanol (%)	TPC (10^6)
Palm juice *C. nucifera*	3.62	17.09	4.63	56.75

Table 1 shown that reducing sugar of palm juice *C. nucifera* L is 17.09 mg/mL.

The reducing sugar in the palm juice *C. nucifera* can be an energy source for indigenous yeast to be converted into bioethanol. It is also seen that amount of cells palm juice *C. nucifera* is 56.75 x 10^6 yeast cells. The cells can convert reducing sugar to bioethanol.

Isolation yeast from palm juice *C. nucifera* L had obtained 5 yeast isolates, namely K3D, K21A, K1C1, K2C, and K1A. The result from screening shown on the Figure 1.



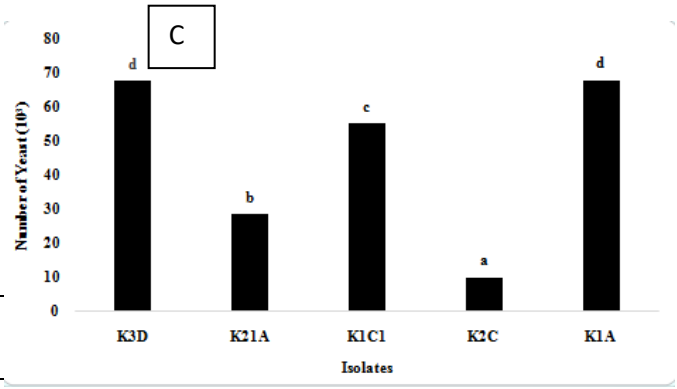


Figure 1. Result screening palm juice *Cocos nucifera* L (A) Ethanol concentration, (B) Reducing sugar concentration, and (C) number of yeast.

On the 6th day the growth and activity of Saccharomyces cerevisiae in the logarithmic, nutritional growth phase consumed and used for ethanol production completely. The speed of the logarithmic growth phase is influenced by the availability of nutrients in the media (Shamim *et al.*, 2016). The lag phase is an adjustment period and 6 days is the optimum time logarithmically or exponentially bioethanol is produced as a primary metabolite, whereas after more than 6 days yeast cells enter the stationary phase and die, so that the bioethanol produced decreases (Apriwida, 2013).

Time of fermentation affect the growth of microorganism. Shorter fermentation time affect inefficient fermentation due to inadequate growth of microorganisms. The other hand, longer fermentation time gives toxic effect on microorganisms growth.

Complete fermentation can be achieved at lower temperatures using a longer fermentation time which produces the lowest ethanol yield. The degree of agitation controls the permeability of nutrients in the cells. The greater the level of agitation, the higher the amount of ethanol produced. In addition, it increases the amount of sugar consumption and reduces the inhibition of ethanol in the cells. The general agitation rate for fermentation by yeast cells is 150-200 rpm. Excessive agitation level is not suitable for ethanol production because it causes limitation of cellular metabolic activity. (Zabed *et al.*, 2016).

By the research, the screening isolates shown that K1A is the best isolate (the highest bioethanol content, the highest reducing sugar, and the highest number of cells), so the K1A isolate was identified used 18S DNA and the result shown Figure 2.

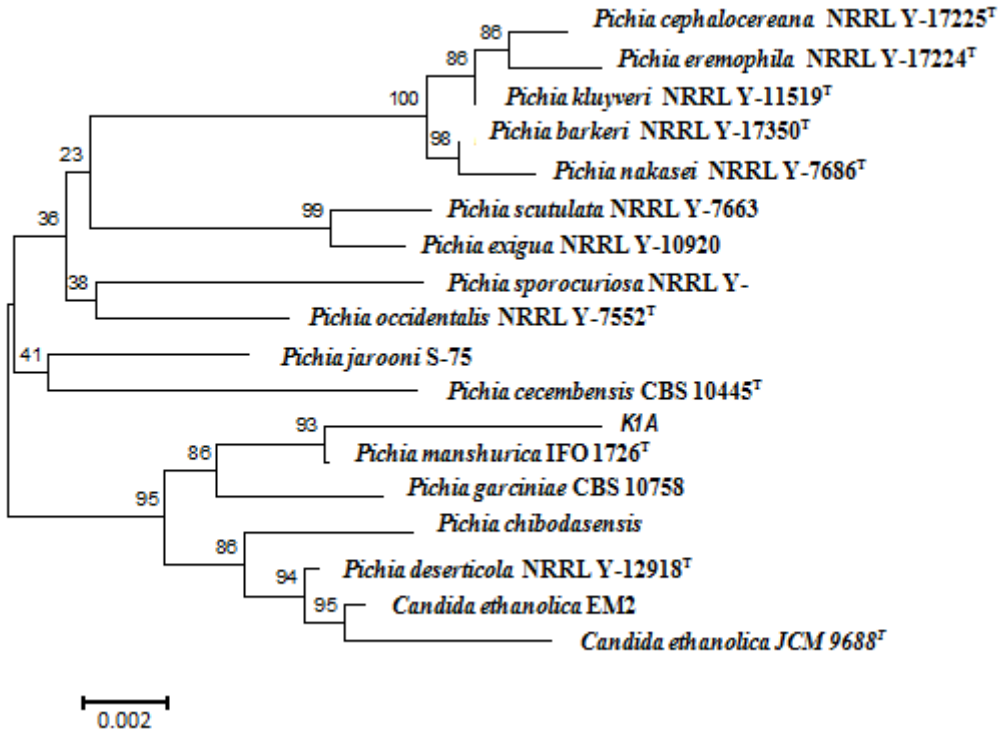


Figure 2. Phylogenetic tree of the K1A isolate based on the 18S rDNA

Table 2. The Identity Similarity Index

No	Species	% Identity
1	K1A	
2	*Pichia manshurica* IFO 10726	99,99
3	*Pichia garciniae* CBS 10758	99,98
4	*Pichia deserticola* NRRL Y 12918^T	99,98
5	*Candida ethanolica* EM2	99,98
6	*Pichia chibodasensis*	99,98
7	*Pichia jaroonii* S-75	99,98
8	*Candida ethanolica* JCM 9588^T	99,98
9	*Pichia sporocuriosa* NRRLY 27347	99,98
10	*Pichia occidentalis* NRRL Y 7552^T	99,98
11	*Pichia scutulata* NRRL Y 7663	99,98
12	*Pichia cecembensis* CBS 10445 ^{T}	99,98
13	*Pichia exigua* NRRL Y 10920	99,98
14	*Pichia barkeri* NRRL Y 17350^T	99,98
15	*Pichia kluyveri* NRRL Y 11519^T	99,98
16	*Pichia nakasei* NRRL Y 7686^T	99,98
17	*Pichia cephalocereana* NRRL Y17225^T	99,98
18	*Pichia eremophila* NRRL Y 17224^T	99,98

IV. CONCLUSION

The candidate for isolates from palm juice *Cocos nucifera* L with the highest bioethanol production was K1A with bioethanol content of 16 %. The results of the identification showed that the isolate was similar to *Pichia_manshurica*_strain:IFO_10726 with 99.99 % similarity value.

V. ACKNOWLEDGEMENT

The authors would like to acknowledge to Ministry of Research, Technology and Higher Education Republic of Indonesia (KEMENRISTEKDIKTI) for the funding support of the research.

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