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Screening and identification indigenous yeast from neera Siwalan for bioethanol production

Screening and identification indigenous yeast from neera Siwalan for bioethanol production

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Abstract The main energy source commonly used by the community comes from fossil energy, especially petroleum. The energy crisis shows that Indonesia's fossil energy reserves are limited. Based on this fact, it is important to develop alternative energy that is environmentally friendly and sustainable, for example bioethanol. The purpose of this research is to screening and identify indegenous yeast from neera siwalan which is potential for bioethanol production. Isolation of yeast from neera siwalan is done using coconut medium. Purification is done to get one pure isolate. Screening is done by observing several parameters: the level of bioethanol production (using an alcohol meter), the content of reducing sugar with the DNS method, and the number of cells with optical density (600nm). Identification is done using ITS 4 and ITS 5. Based on the results of screening, four yeast isolates were produced, namely S3D, S1A, S2D, S1C. Based on these results the superior isolates producing bioethanol are S1A (14.4%). The results of the identification showed that the isolate was similar to *Candida tropicalis* with 100% similarity value.

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 [1]. However, these sources are no longer considered sustainable, and their availability is much less. Shafiee and Topal [2] predicted that oil, coal and gas would only be sequentially left around 35, 107, and 37 years. In addition, these fuels cause environmental impacts such as global warming due to greenhouse gas emissions. Therefore, there is a requirement for renewable energy sources, sustainable, and environmentally friendly, bioethanol is one of them.

Bioethanol is one of the main renewable energy sources which is undoubtly, a future fuel. Bioethanol has higher octane number and relative to that of gasoline alone, its use as a blender with

gasoline reduces the emission of CO_2, NO_x and hydrocarbons after combustion. The use of ethanol shows high compression ratio and increased energy production in combustion engine [3] .

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 [4-9]. Bioethanol is generally produced with the favour of yeast type microorganisms with simple sugar carbon sources from molasses (molasses), corn or sugarcane [10] Other than molasses, another ingredient that can be used as a source of bioethanol is neera. Neera is a sweet liquid containing sugar at concentrations of 7.5 to 20.0% contained in the flowers of sugar palm, coconut, and palm trees whose buds have not been bloomed and obtained by means of tapping [11]. Neera contains water 87.66%, sugar 12.04%, protein 0.36%, fat and ash 0.36% and 0.21%, respectively [12].

Various researches have been carried out for producing ethanol from several biomasses include micro and macroalgae [13]; molasses [14, 15]; coconut neera [16]; aren neera [17], nypha neera [18, 19]; and *Sargassum* [20, 21]. The previous studies related to molasse and micro and macroalgae algae as a material of producing ethanol by utilizing *Saccharomyces cerevisiae* with ethanol content ranging from 2.709% - 94%. The purpose of this research is to screening and identify indegenous yeast from neera siwalan which is potential for bioethanol production.

2. Materials and Methods

2.1. Sampling of neera and isolation of yeast

Neera Siwalan taken from Rembang Central Java. These neera was taken each of 250 mL (3 replicates) in a bottle and put in a cooler to be brought to the laboratory. Samples of 25 mL of samples plus 225 mL of physiological salt (10^{-1}) were made series dilution to 10^{-9}. The samples suspension at each dilution was taken 0.1 mL inserted into a sterile Petri dish and poured 15 mL of medium YEPD to be pourplate and homogenized. The culture was incubated at 25 °C for 48 hours. Each type of yeast colony that is grown was calculated and then purified.

Each yeast colony is purified by the spread plate method. The unit of yeast colony was put into 10 mL of physiological salt then made series dilution to 10^{-6}. Suspension of yeast culture taken 0.1 mL then spread (spread) with Drigalski glass rods on the surface of the medium to make YMEA in Petri dish. The yeast culture was incubated at 25 °C for 48 hours [22]. In this research, the characterization of samples includes pH and sugar content. The data was tabulated and analyzed by α: 5% variety with SPSS program. The pure yeast stock then was screened to obtain the highest potential isolates on ethanol production.

2.2. Screening yeast ethanol producer

This study used Completely Randomized Design (RAL) with yeast isolate, and incubation time. Parameters observed included reducing sugars, yeast cell counts, and ethanol content. The action step begins with the removal of pure isolates of 1 ose grown in 100 mL subcultured medium incubated 24 h. Prepared 12 bottles media of neera siwalan each 100 mL and sterilized with autoclave 121 °C for 15 minutes and cooled. Taken 10% (10 mL) each of subculture media with pure isolates and put into sterilized media aseptically then fermented with a time of 0, 2, 4, and 6 days, and measured reducing sugars with the DNS method, the number of cells with OD (600nm), and bioethanol levels with Alcohol meter. Each treatment repeated 3 times [22]. The data obtained were tabulated and analyzed varieties α: 5% using SPSS program version 16. If treatment gave significant effect followed by DMRT real difference test. Based on the DMRT test, the selected isolates were obtained.

2.3. Identification of yeast based on ITS sequences

2.3.1.Chromosomal yeasts DNA extraction

DNA extraction was done by Sambrook and Russell [23]. 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 µL lysozyme (50 µg / mL) then shaken to homogeneous and incubated 37 °C for 30 min. To dissolve the membrane and enzyme proteins, GES reagent was added as much as 250 µL, homogenized until completely dissolved and incubated for 10

min at room temperature. Supplement plus 125 µL ammonium acetate 7.5 M and placed in ice for 10 minutes. The separation of DNA from proteins and polysaccharides was done by adding 500 µL chloroform to the solution, flipped 50 times, and centrifuged at 10,000 rpm for 10 min. Once completed centrifuse 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 Ependrof. To form DNA threads, into the solution DNA isopropanol is added 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 µL 0.2X TE buffer, and then the DNA concentration was measured using spectrophotometer at 260 nm wavelength.

2.3.2. Sequence amplification of ITS with PCR

Sequences of ITS were amplified using a general primer ITS 1 (5´TCC GTA GGT GAA CCT TGC GG 3´) and ITS 4 (5´TCC TCC GCT TAT TGA TAT GC 3´) [24]. Amplification was performed on a 25 µL µL reaction mixture containing 19 µL sterile water, 25 green GoTaq (Promega), 2 µL ITS 1, 2 µL ITS 4, 2µL DNA. Amplicon was amplified under PCR conditions 94 °C for 3 min (initial denaturation), continued (94 °C, 1 min denaturation, 50 °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 [25].

2.3.3. Sequencing and BLAST analysis

The purified PCR result then disequens using automatic sequencing mach ABI 3130 XL Genetic Analyzer using primer ITS 1/ ITS 4. The result is analyzed by BLAST at www.ncbi.nlm. nih.gov to identify closely related species of Saccharomyces [25].

3. Results and Discussion

Based on the sampling of neera Siwalan the initial condition of these shown on Table 1

Table 1. The condition of neera Siwalan.

	pH	Sugar reduction (mg/mL)	Level ethanol (%)	Total Plate count (10^6)
Neera Siwalan	4.39	43.35	2.13	38.3

Based on Table 1, it can be seen that neera Siwalan contains high reducing sugar, which is 43.35 mg/mL. The reducing sugar can be an energy source for indigenous yeast neera Siwalan to be converted into bioethanol. It is also seen that neera Siwalan contained 38.8 x 10^6 yeast cells. These indigenous yeast cells can convert reducing sugars to bioethanol.

Isolation yeast from neera Siwalan which was carried out based on Blanco et al. [22] had obtained 11 yeast isolates, and based on the shape and character of yeast had obtained 4 isolates, namely S3D, S1A, S2D, and S1C. Screening is done by observing several parameters: the days o fermentation (0,2,4,6 days), the level of bioethanol production (using an alcohol meter), the content of reducing sugar with the DNS method, and the number of cells with optical density (600nm). The result from screening shown that the highest bioehanol with the 6 days fermentation shown in these Figures.

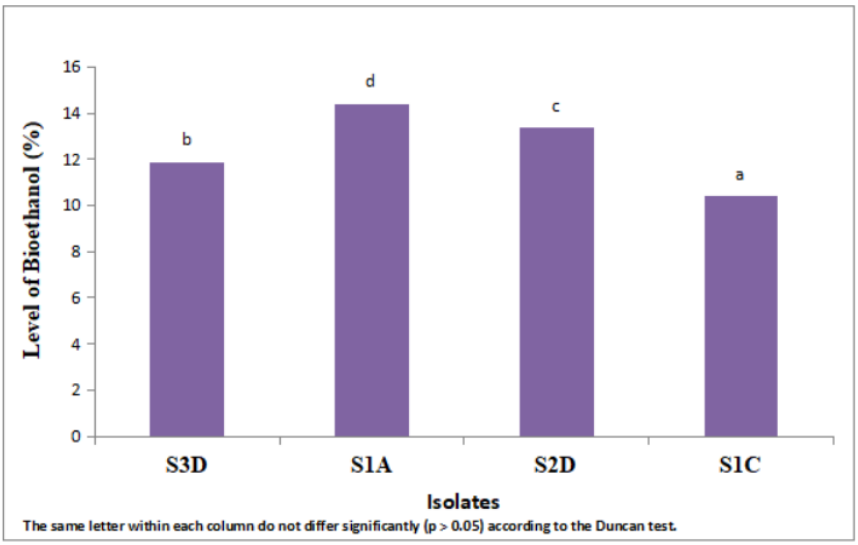

Figure 1. Level of bioethanol.

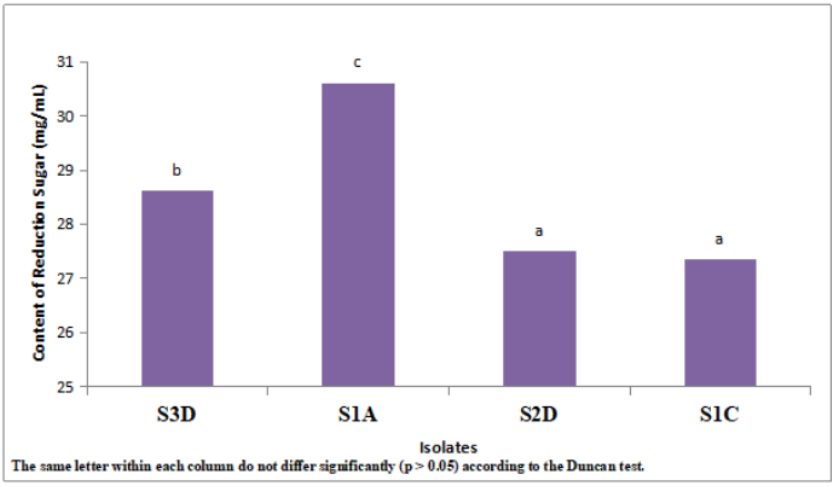

Figure 2. Content of reduction sugar.

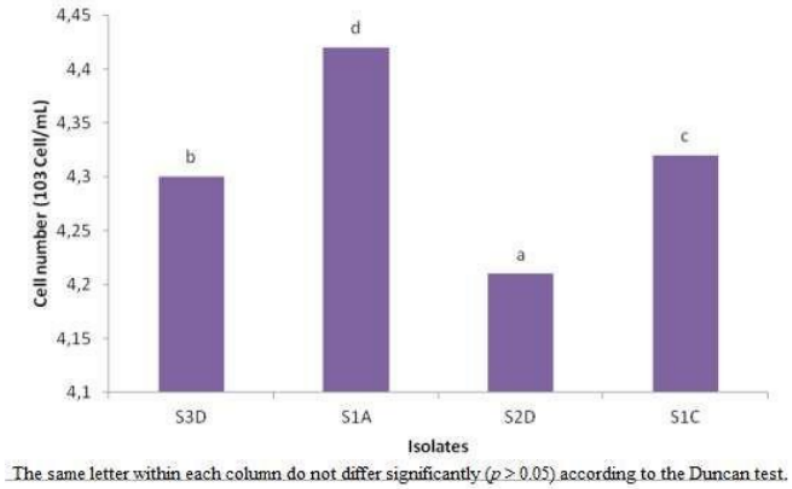

Figure 3. Cell number (10^3 Cell/mL).

The days of fermentation with the highest bioethanol fermentation is 6 days, because. the days time it is entering an exponential phase where the number of microbes of yeast and enzymes is secreted at the optimum amount. The longer the fermentation process, the activity of yeast as microbes which become degrading agents of sugar into bioethanol is also decreasing [26]. The lag phase is the adjustment period and the time of 6 days is the optimum time which is the exponential or logarithmic which that bioethanol as the primary metabolite is produced, whereas after more than 6 days yeast cells enter the stationary phase and death, so that the bioethanol produced decreases [27].

Based on these screening shown that the highest level of bioethanol production, the content of reducing sugar, and the number of cells is S1A isolat. The next step is identification isolat S1A used ITS 1 and ITS 4. The result shown these Figure 4.

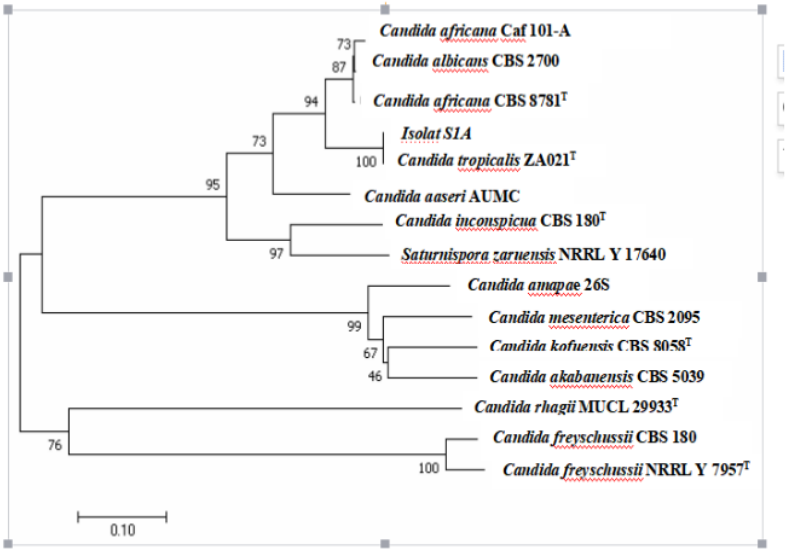

Figure 4. Phylogeny tree the S1A isolate reference *Candida tropicalis* on Neighbor-Joining Algorithm.

4. Conclusion

The candidate for isolates from neera siwalan with the highest bioethanol production was S1A with bioethanol content of 14.4%,. The results of the identification showed that the isolate was similar to *Candida tropicalis* with 100% similarity value.

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