Selection and characterization yeast potential from pineapple for bioethanol production using some sugar sources

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Abstract. Bioethanol can be produced through fermentation process of sugars based on carbohydrate biomass such as lignocellulosa by microorganism such as yeast. Yeast changes sugar as a carbon source to produce ethanol and CO$_2$ in anaerobic condition. Pineapple as a fruit commodity that is widely cultivated in Indonesia contain high sugar and carbohydrates so it might contain yeast which has the potential in bioethanol production. This research aims to select and characterize potential yeast from pineapple for bioethanol production from some sugars. The method included fermentation test, ethanol production (measurement of reduction sugar, dry cell biomass, and ethanol contain), and molecular identification. Yeast utilize substrates as carbon source for producing cell or ethanol. Ethanol production influenced by substrates, microorganism, and total cell. The results showed that NHC3 used as selected isolate based on the degree of coloration in Oxidative Fermentative Media. The largest ethanol production was 10.63 g/L obtained from mannose where the highest value of substrate utilization efficiency was 95%. The value of yield product is 0.27 g bioethanol/ g substrate and yield biomass is 0.14 g biomass/ g substrate. BLAST result shows NHC3 had homology >99% with Saccharomycetales and Kodamaea ohmeri.

Keywords: Bioethanol, fermentation, sugars, yeast, pineapple

1. Introduction

Ethanol (C$_2$H$_5$OH) originates from the carbohydrate fermentation process using microbes, because the manufacture involves biological processes, so the ethanol product produced is named bioethanol. Bioethanol is obtained from the fermentation of sugar-containing materials and then the distillation process is carried out to obtain pure alcohol [1]. Ethanol has many physical and chemical properties that can be used for industrial raw materials, disinfectant materials, solvents, and bioenergy [2]. Bioethanol grade must be different according to its use. Bioethanol has a grade of 6.5% - 90% volume can be used in industry, grades of 96% - 99.5% are used in mixtures for alcohol and pharmaceutical ingredients. Bioethanol can replace the function of additives that are often added to increase the octane value of fuel. Another advantage of bioethanol productions is that it is a simple way of making fermentation using certain microorganisms [3].

Generally bioethanol is made from carbohydrate-rich plants such as yams, cassava and corn. However, these plants have other use values as food, which creates a contradiction to food needs when applied in developing countries such as Indonesia. In addition, the problem of developing bioethanol is at relatively high production costs. Several attempts were made to reduce production costs, including the selection of cheaper production media. Lignocellulosic biomass including agricultural, plantation
and forestry waste is very abundant and is an economical source of energy. The waste is collected every year in large quantities and causes environmental problems [4]. The main components in lignocellulose are cellulose, hemicellulose, and lignin. All three form a complex chemical bond that becomes the basic material of plant cell walls. Hemicellulose is the second most common polysaccharide in nature, which ranges from 20-35% and consists of pentose (xyllose), hexose (manose, glucose and galactose), and other monosaccharides such as fructose [5]. The sugar components are components that are widely used by microorganisms during the fermentation phase. Based on the constituent compounds of biomass, biomass can be used to produce products that have added value such as ethanol.

Ethanol is generally produced through fermentation of ethanolological microorganisms such as yeast [6]. Yeast is non-pathogenic, non-toxic, and has several important enzymes such as phosphatase, lipase, zimase and proteinase, which cause yeast to play an important role in the decomposition of organic compounds such as fermentation so that it is widely used for industrial use [7]. Yeast is an important role in the bioethanol industry that uses sugary substrates. Carbohydrates will be hydrolyzed into sugar monomers and yeast will metabolize sugar to form pyruvic acid through the stages of reaction in the Embden-Meyerhof-Parnas pathway, while pyruvic acid produced will be dioxobylated to acetaldehyde, which then dehydrogenates into ethanol [8]. While yeasts that actively produce alcohol can be isolated from natural substrates containing sugar including pineapple.

Pineapple plants (Ananascomosus (L) Merr) are one of the many plant crops grown in Indonesia. There are several types of pineapple varieties, including Smooth Cayenne, Queen and Spanish. There are 2 types of Spanish, namely Red Spanish and Green Spanish. The varieties widely cultivated by Indonesian farmers are Smooth Cayenne and Queen varieties [9]. Pineapple has many benefits as food, feed and industrial raw materials. The prospect of pineapple agribusiness is very bright, tends to increase both for the needs of fresh fruit as well as processed ingredients. The main part of the important economic value of pineapple plants is the fruit which has high carbohydrate (oligosaccharide) and glucose (monosaccharide) content [10].

According to Anggraini [11], pineapple skin contains 81.71% water, 20.87% crude fiber, 17.53% carbohydrate including reducing sugars by 13.65% and 4.41% protein whereas pineapple fruit per 100 g containing total carbohydrates of 12.63 g with sugar content of 9.26 g and dietary fiber of 1.4 g so that the carbohydrate and sugar content that high enough in pineapple can contain yeast which has the potential in bioethanol production.

2. Methods

2.1. Isolate rejuvenation
Rejuvenation was carried out by growing isolates on YPDB (Yeast Peptone Dextrose Broth) media and incubating for 24 hours. The isolates were then transferred to the YPDA (Yeast Peptone Dextrose) media with a quadrant scratch method and incubated for 24 hours. The single colonies produced were grown on the media to be slanted and incubated for 24 hours. Isolates were isolated from pineapple fruit.

2.2. Yeast selection based on ability in using carbon sources
Yeast selection done by conducting a fermentation test using oxidative fermentative media refers to Hugh and Leifson [12]. Isolates were inoculated into YPDB media for incubation for 24 hours. Optical Density (OD) values were measured by a spectrophotometer at a wavelength of 600 nm. The magnitude of the measured OD will determine the volume of isolate culture that must be included in the oxidative fermentative (OF) media. The cultures were then inserted and incubated in OF media supplemented with various sugar substrates (fructose, galactose, glucose, mannose, and xylose). Incubation was carried out for 48 hours at room temperature. Media color changes from blue to yellow indicating a decrease in the pH of the medium due to the formation of acidic compounds from
fermentation were observed after 48 hours of inoculation and incubation. The test done by doing two replications. The best fermented yeast was used as the selected isolate.

2.3. Measurement of reducing sugar content
The ability of yeast to use sugar substrate is closely related to its ethanol production, therefore, reducing sugar content measured by the dinitrosallic acid (DNS) method [13]. DNS reagent (1 L) consists of 6.3 g DNS, 182 g Rochelle salts (Potassium sodium tartrate), 5 g phenol, 5 g sodium bisulfite, and 21.4 g sodium hydroxide. Measuring sugar levels was carried out by taking 0.5 mL of sample and then put it into a micro tube volume of 1.5 mL and centrifuged 8,000 rpm for 10-15 minutes. The supernatant was taken and then added distilled water with a total volume of 1 mL (according to the required dilution) and 2 mL DNS. Saving the reaction was then vortexed and heated at 100°C for 10 minutes to react between sugar and reagent DNS. The tube cooled at room temperature and measured by a spectrophotometer at a wavelength of 540 nm.

2.4. Measurement of total biomass
Biomass measurement was done by determining the weight based on the dry weight of biomass contained in the liquid during cultivation. A total of 1.5 ml sample was inserted into the microtube, which had previously been known for its weight. The sample was then centrifuged at 8,000 rpm for 10 minutes. Separation was carried out between solids (biomass) and the supernatant. Microtube containing solids were washed using 5 ml sterile aquades, then centrifuged again. Separation carried out again between solids and distilled water. The solids were dried in an oven at 50°C for 24 hours or the biomass weight was constant. The weight of dry biomass was the weight of the tube containing dried biomass minus the initial tube weight [14].

2.5. Measurement of ethanol levels
The alcohol testing procedure carried out by the distillation method according to the instructions of Azizah et al. [15]. The distillation process carried out to separate ethanol from the fermented solution by heating the solution at the ethanol boiling point of 78ºC, so that ethanol first evaporated and then channeled to the pipe, condensed and returned to liquid ethanol. A sample of 200 ml was put into a flask and distilled at 80ºC. Destillate was stored in erlenmeyer to a volume of 100 ml. The distillate then put into a measuring cup then the ethanol level was measured using alcoholmeter.

2.6. Molecular identification of selected yeast isolates
Selected yeast isolates were identified molecularly based on the internal transcribed spacer (ITS) sequence [16]. The isolation of genomic DNA was carried out using YeaStar Genomic DNA Kit. The measurement of the absorbance value of the quality and quantity of DNA was carried out by Thermo Scientific Nanodrop 1000 spectrophotometer. Measurements for DNA concentration were carried out at wavelengths of 260/280. The primers used for PCR reactions were ITS1 (5’-TCC GTA GGT GAA TGT GG-3’) and ITS4 (5’TCC TCC GCTTA TTG TGC-3 ‘ATA) [14]. A mixture of 50 µL of the PCR reaction was arranged as follows: 11 µL nuclease free water, 5 µL primer ITS1, 5 µL primer ITS 4, 4 µL DNA template (87.1-100.7 ng/µL), and 25 µL GoTaq® Green Master Mix. The PCR condition was regulated under the following conditions: pre-denaturation at 94°C for 4 minutes in 1 cycle; denaturation at 94°C for 30 seconds, annealing at 49°C for 30 seconds, elongation at 68°C for 1 minute for 30 cycles; ended with post-elongation at 68°C for 20 seconds and cooling at 15°C for 5 minutes in one cycle.

The PCR product was visualized using electrophoresis in a 1% agarose gel at a voltage of 100V for 25 minutes, with a marker of DNA marker size of 1 kb. Electrophoresis agarose gel soaked in EtBr for 10 minutes then was rinsed with distilled water then observed on UV light. Furthermore, PCR products were sent to the sequencing service agency. Analysis of sequencing results were begun with a sequence of DNA base sequences of ITS regional codes from forward sequences and reverse sequences using the Mega 6 application. Sequences that have been contested are aligned with
sequence data contained in the GenBank database using the Basic Local Alignment Search Tool (BLAST) nucleotide, which integrated with NCBI [17]. Alignment results were used for species identification. The stages were carried out in the Division of Genetic Laboratory, PT. Genetics Science Indonesia, Jakarta.

3. Results and discussion

3.1. Yeast selection based on ability in using carbon source

Fermentation and oxidation play an important role in the process of metabolizing microorganisms. This test was conducted to determine the ability of yeasts to use carbon substrates (sugar) by fermentation. This study used a carbon substrate (sugar) in the form of fructose, galactose, glucose, xylose, and mannose. Yeasts which potential for the bioethanol industry must have the ability to do the fermentation and produce bioethanol. The test results using OF media can be seen in table 1. The fermentation results showed that almost all the final pH of the fermentation medium had a decrease in pH values (figure 1).

Table 1 Color changes in OF media with various carbon sources after 48 hours fermentation by several isolates

| Substrates | Isolates     |
|------------|--------------|
|            | NH3 | NHC2 | NHC5 | NHC3 | NHC1 |
| Fructose   | ++++| ++++| ++++| ++++| ++++ |
| Galactose  | ++  | +   | +   | ++  | +   |
| Glucose    | +++ | ++++| ++++| ++++| ++++ |
| Mannose    | ++++| ++++| +++ | ++++| ++++ |
| Xylose     | ++++| +++ | +++ | +++ | +++ |

(+) Indicates that there are color changes from blue to yellow, the more + the more yellow.

![Figure 1](image) Measured pH of OF media with various kinds of sugar substrate after 48 hours incubation by NHC3 isolate.

3.2. Measurement of reducing sugar content

Hydrolysis of complex carbohydrates will produce simple sugars. The availability of reducing sugars in the bioethanol production medium is one of the important elements for yeast growth because it
serves as a carbon source for energy formation. The sample sugar concentration tested can be
determined by entering the absorbance of the sample obtained into the linear line equation of standard
solution. The measurement of the initial reducing sugar concentration ($S_0$) in all samples decreased
after incubation ($S$). The yeasts had the ability to use substrates for the formation of biomass and
products based on the value of substrate utilization efficiency ($\Delta S / S_0$). NHC3 isolate had a high
efficiency of utilization of large substrates (93% -95%) on fructose, glucose, and mannose substrates
(figure 2).

![Substrates Utilization Efficiency](image)

**Figure 2.** Efficiency of each substrate utilization ($\Delta S/S_0$) by NHC3 isolate.

3.3. *Measurement of total biomass*
Sugar substrate as a carbon source can be use by yeast as an energy source, biosynthesis, and growth
factor. The yeast growth was characterized by the increasing amount of biomass during fermentation.
The calculated biomass was the weight of dry biomass in fermented liquids. Figure 3 shows the
measurement of NHC3 isolate biomass in each sugar substrate treatment. The highest cell biomass
was produced by NHC3 isolate with mannose substrate of 5.63 g/L while the lowest NHC3 isolate cell
biomass was produced on xylose substrate of 0.63 g/L.

![Cell Biomass](image)

**Figure 3.** Dry cell biomass NHC3 isolate in various sources of sugar after incubation 3 days at room temperature.

3.4. *Measurement of ethanol levels*
The distillation process carried out to separate ethanol based on its boiling point. The results of the study can be seen in figure 4. The greater the ethanol content produced, the less total sugar remaining. The largest ethanol content was obtained from mannose substrate reaching 10.63 g/L. Ethanol obtained from four sources of sugar, namely glucose, fructose, galactose, and xylose. This was also in accordance with the decrease in sugar content. Growth and formation of products by microbes is a process of bioconversion of substrates in fermentation into mass cells and or metabolite products. The important parameters that illustrate the efficiency of substrate conversion into biomass or products other than P (product) and substrate efficiency ($\Delta S / S_0$) are yields of biomass ($YX / \Delta S$) and product yield ($YP / \Delta S$) that were presented in figure 5.

![Figure 4](image1.png)

**Figure 4.** Ethanol levels NHC3 isolate for each type of sugar.

![Figure 5](image2.png)

**Figure 5.** Yield of ethanol for each substrate ($\Delta YP$: yield product, $\Delta YX$: yield biomass).

3.5. *Molecular identification of selected yeast isolates*

NHC3 isolate was molecularly identified with the aim of knowing their species. The results of DNA amplification showed DNA bands at a length of base pair ± 450 bp (figure 6). PCR products were then sequenced to determine the nucleotide sequence of NHC3 isolates. The results of the sequencing stage were data sequences consisting of sequences of bases. Sequence data could be used to find the identity...
of an isolate by searching for the closest species sequence homology in the data sequence in the database. Sequence homology searches use the BLAST program by accessing the database site address such as http://www.ncbi.nlm.nih.gov/ (table 2).

Figure 6. Results of amplification of ITS fragments from the genome of NHC3 isolate using primary pairs of ITS1 / ITS4. (M: DNA marker measuring 1 kb).

Table 2. Analysis of BLASTN homology of selected isolates based on ITS rRNA sequences.

| Isolate Code | Homology               | Max Score/Total Score | Query Cover(%)/Identity (%) | E-value | Accession Number       |
|--------------|------------------------|-----------------------|-----------------------------|---------|------------------------|
| NHC3         | Saccharomycetales sp.  | 783/783               | 100%/99.76%                 | 0.0     | gi|117653097|EF0606092.1 |
|              | Saccharomycetales sp.  | 773/773               | 100%/99.76%                 | 0.0     | gi|117653066|EF060661.1  |
|              | Kodamaea ohmeri voucher MCCC2E00326 | 752/107/5       | 100%/99.76%                 | 0.0     | gi|124126992|EF196811.1  |
|              | Kodamaea ohmeri CBS 5367 | 747/747              | 100%/99.51                  | 0.0     | gi|662009444|NR_121464.1 |
|              | Kodamaea ohmeri isolate WM10.200 | 743/743            | 100%/99.27                  | 0.0     | gi|357057414|JN183447.1  |

4. Discussion
Each yeast isolate had different abilities in using substrate sources depending on the metabolic pathway. In general, all isolates had the ability to use various carbon sources tested. However, the level of fermentation carried out by each isolate are different. NHC3 isolate had good fermentation
ability for all sugar substrates compared to other isolates so NHC3 isolate was used as test isolates based on the color change rate in Oxidative Fermentative media. Changing of the color of the medium to yellow due to the presence of indicators of Bromthymol Blue (BTB) in the medium. Increasing or not the pH of the medium could be seen from the color changes that occurred. BTB color indicators are yellow in acidic conditions and will turn blue in neutral conditions and purplish blue in alkaline conditions [16]. Fermentation causes the color change of the media to turn yellow. The increasingly yellow color changes according to Altschul et al. [17] the higher ability of yeast to use carbon sources so that pH becomes acidic.

The BTB indicator will change the medium to yellow because the media pH decreases (figure 1). Hugh and Leifson [12] mention that the results of CO₂ gas from fermentation will form H₂CO₃ acid if it reacts with water, so CO₂ also contributes to a decrease in pH. This decrease in pH was also caused by the accumulation of organic acids produced in fermentation. The acid will then be converted by yeast to ethanol [18]. In addition, according to Wurts and Durborow [19] during the fermentation process there are two moles of H⁺ ions released when glucose are broken down into two moles of pyruvic acid, so that the accumulation of H⁺ ions affects the decrease in pH in the media.

Yeast potential for the ethanol production industry must have the ability to ferment carbon sources to produce ethanol. This was tested by observing media sugar levels using the DNS method. The decrease in total sugar, especially in three test substrates supported by several yeast isolates isolated from cocoa plants [20] that had high efficiency using glucose, fructose, and mannose sugars compared to other substrates. Based on Stanbury and Whittaker [21], the fermentation process that produces ethanol will reduce total sugar levels in the medium. Yeast used sugar so that there was a decrease in sugar levels in the media. The higher the decrease in sugar levels should indicate the higher the ethanol content produced by yeast, the lower the reduction in reducing sugar levels, the lower the ethanol content produced by yeast [22].

The lowest efficiency of substrate utilization on the xylose substrate was caused by the low amount of substrate consumed by NHC3 isolate. According to Bayu et al. [23], xylose, which is pentose sugar, has a pentose phosphate pathway so it could not be used as a carbon source. This can make competition between yeasts due to the limited availability of substrates. Such competition can cause the death of several cells, thus allowing efficient substrate utilization by yeast [24].

Efficiency of substrate utilization shows the ability of yeast to use substrate for the formation of biomass and / or products. The efficiency of substrate utilization on glucose, fructose and mannose substrates reached more than 90%. These results indicate that the utilization of glucose, fructose, and mannose in forming cell biomass or products are relatively higher. The low efficiency of utilizing the substrate on xylose and galactose sugars will affect the ethanol yield value on both substrates.

At the time of fermentation, yeasts first experience a growth period before they are ready to hydrolyze sugar into alcohol. The initial growth was characterized by the addition of cell mass then the cells divided rapidly until the population was large and ready to hydrolyze into alcohol [25]. The largest ethanol content obtained by the mannose substrate caused by the largest amount of biomass compared to other substrates. This was consistent with the results of Suarez-Mendez et al. [26] which states that the more cells there were, the more sugar will be converted into ethanol.

Biomass production influenced by the type of substrate used and the ability of yeast to consume the substrate. The highest cell biomass was produced by NHC3 isolate with mannose substrate. These results appropriate with the research of Anggraini [11], which showed that yeast is easier to use hexose sugar than other types of sugar related to the complexity of the sugar structure and its metabolic pathway. The lowest biomass was obtained on the xylose substrate. The results of Gunam et al. [27] also showed that Saccharomyces cerevisiae did not produce ethanol on the xylose substrate. S. cerevisiae is only able to convert hexose sugar to ethanol while xylose is pentose sugar. This happens because the enzyme S. cerevisiae takes longer to convert xylose sugar to ethanol. Xylose which is pentose sugar will be converted to ethanol through the pentose phosphate pathway while hexose sugar through the Enter Duodroroff Pathway. Subtil and Boles [28] also stated that yeast growth in disaccharides, oligosaccharides, and polysaccharides requires a system of metabolism in the form of
enzymes. These enzymes require an induction time during growth so that not all monomers can be metabolized directly from yeast to form ethanol.

Product yield (YP / ΔS) shows the utilization of the substrate that is converted into products while the yield value of the biomass (Yx / ΔS) shows the utilization of the substrate into cell biomass. The results showed that all substrate had a value of YP / ΔS which was greater than the value of Yx / ΔS (figure 5). This showed that yeast used more of the substrate to form ethanol rather than forming cell biomass. The xylose substrate had the lowest YP / ΔS and Yx / ΔS values. It was because of low levels of ethanol on the xylose substrate (figure 4) so that the xylose substrate did not efficient in making bioethanol.

The mannose substrate with the highest ethanol content also had the highest YP / ΔS value with a large Yx / ΔS value. This shows that the mannose substrate had the highest efficiency of utilization by yeast in the formation of bioethanol. The yield value of the product obtained by mannose. Based on Kumar et al. [29], if the yield of ethanol obtained below 0.51 then there are another form of substrate conversion. Other forms of substrate conversion include biomass and other organic compounds. This appropriate with the results that there was a substrate utilization to form cell biomass (figure 2), whereas if ethanol yield above 0.51, there is a number of substrate for ethanol and biomass production that were not measurable.

Griffin [30] stated that isolates that had sequence homology of ITS region> 99% with the closest species were the same species, whereas isolates that had ITS area sequence homology <99% with the closest species were different species from the species in the GenBank database. This shows that the sequence of nucleotide isolates is identical to the nucleotide sequence in the GenBank database so that it indicates the same species.

The BLASTN analysis table lists items or records based on their homology level with sequences analyzed, starting from the highest level to the lowest level. The parameters that appear in the BLASTN analysis are the total score which shows the total base pair value, the max score which shows the similarity (identical) base pairs, the query cover that shows the percentage of nucleotide samples used in the BLASTN analysis, and identity which shows the percentage of identification accuracy. The higher the value of max score and identity, the higher level of homology e-value is a statistical probability level for a record to be similar to a query that is analyzed or can also be interpreted as a percentage of errors so that the smaller the e-value the higher the homology level. Asesi is an accession number or special numbering for each record [31].

The results of homology analysis of selected yeast isolates using the BLASTN program compared with GenBank data showed that NHC3 isolate had homology of> 99% with Saccharomyces (table 2). Saccharomyces. According to Sugita et al. [32], Saccharomyces sp. and Pichia sp. are types of Saccharomyces including industrial yeast which is often used in bioethanol production. Based on Nugraha et al. [33], Saccharomyces cerevisiae NCIM 3570 yeast can produce ethanol with an ethanol concentration of 9.83 ± 0.20 g / L, but ethanol from xylose media did not produced. Ethanol produced by S. cerevisiae was greater than that of Pichia stiptis NCIM 3499 with ethanol yields of 3.97 ± 0.21 g / L and 9.94 ± 0.19 g / L for 40 hours fermentation time, respectively. The method of combining S. cerevisiae and P. stiptis conducted by Daud et al. [34] using lignocellulose media can also produce ethanol yields of 0.5 g/g.

**Conclusion**

The potential yeast for the bioethanol industry must have the ability to do fermentation to produce bioethanol. NHC3 isolate had good fermentation ability on all sugar substrates compared to other isolates so that NHC3 isolate were used as selected isolates based on the degree of discoloration in Oxidative Fermentative media. Furthermore, ethanol production influenced by the type of substrate and number of cells. The largest ethanol content was obtained by manosa substrate of 10.63 g/L with the highest substrate utilization efficiency reaching 95%, the highest product yield value reached 0.27 g of bioethanol / g substrate and yield of cell biomass of 0.14 g cell biomass / g substrate. BLAST results showed NHC3 isolate had homology> 99% with Saccharomyces sp and Kodamaea ohmeri.
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