Screening and characterization of potential bioethanol production yeast from tropical fruits

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Abstract. This study aims to separate and characterise indigenous yeast (IY) from tropical fruit waste. The techniques include isolating and characterising yeast from different kinds of fruit waste, testing yeast for ethanol and glucose tolerance, and producing bioethanol in vitro. Using a microscope and visual inspection, the yeast's morphological identification is done. Using a spectrophotometer to measure optical density, the tolerance tests for glucose and ethanol are used to select yeast biochemically. With the Gas Chromatography-Flame Ionisation Detector (GC-FID), one can measure the amount of ethanol present. Yeast was isolated using selective media to yield six isolates: code A1 from grapes, codes NG1, NG2 from jackfruit, and codes N1, N2, and N3 from pineapple; mango produced no results. Three isolates with the codes A1, NG1, and NG2 were chosen based on test results for resistance to glucose and ethanol. The *Saccharomyces cerevisae* bioethanol production test yielded 6.60%, 3.30%, 4.5%, and.4.85% of ethanol for the yeast species coded A1, NG1, and NG2, respectively, in terms of ethanol. According to the study's findings, yeast bearing the NG2 code may be used in the fermentation process to produce bioethanol.

Keywords— bioethanol, yeast, tropical fruits.

1. Introduction

According to recent estimates, there is a growing shortage of fuel in the world, necessitating the use of alternative energy sources to replace fossil fuels. Using bioethanol or other alternative energy sources can help solve this issue. A compound known as bioethanol is produced when microorganisms use fermentation to convert sugar from carbohydrates like starch, cellulose, and glucose into alcohol. Saccharomyces cerevisiae is the most commonly used microorganism in the alcoholic fermentation process. This yeast is extremely reproducible, resistant to high sugar levels, tolerant of high alcohol concentrations, and active between 4 - 32^{o} C [1]. The type of yeast is one of the variables that affects the amount of bioethanol produced. Therefore, in order to identify isolates that have the potential to produce bioethanol, yeast must be isolated and identified. Yeast is present in a variety of habitats, particularly those with high sugar content, like fruit. [2]

The process by which cells produce energy in anaerobic environments is called fermentation. Anaerobic respiration, or respiration in an anaerobic environment with an external electron acceptor, is generally referred to as fermentation. Because sugar is a source of energy in fermentation, the kind of sugar used will determine the variations in fermentation reactions. The simplest sugar, glucose ($C_6H_{12}O_6$), ferments to produce two molecules of ethanol (C_2H_5OH). Yeast is the agent of this

fermentation reaction. Chemical reaction equation: $C_6H_{12}O_6$ $2C_2H_5OH + 2CO_2 + 2$ ATP which initially disaccharide sugar will be broken down into monosaccharides first [3], [4]

A unicellular microorganism with a broad range of habitats is yeast. Yeast has a high organic matter content and can survive in harsh conditions. Yeast that produces bioethanol can be found in places with high levels of organic matter. [5]. Soil and plants contain highly organic materials; one ingredient with a high organic content is fruit. In this study, pineapple, grapes, and jackfruit were the selected tropical fruits. They have a fairly high sugar content and are commonly available in tropical regions. [4]

One method to identify unknown types of yeast is to characterise and isolate the yeast. Both morphological and biochemical tests are used in this identification process.[6]. These findings are also used to identify the isolate's genus, but if genetic testing is successful, the isolate may be elevated to a new species level. Macroscopic and microscopic structural observations were used to conduct morphological tests on yeast colonies and cells. We will compare the macroscopic and microscopic test results with yeast strains that are known to exist. If the goal is to determine the species level, these results are still weak. Based on the morphological similarity between unknown isolates, the morphological test can be used to estimate the genus of those isolates [7].

The morphological tests must be strengthened with yeast biochemical testing. Throughout its life, yeast exhibits distinct biochemical metabolic properties. Isolates whose species are unclear down to the genus level can be interpreted by the biochemical test. The quality of the yeast determines its ability to be used successfully in industrial products. Searching for species with high ability or tolerance to conditions that frequently arise in industrial processes is therefore necessary [8] ,[9]. Resistance tests on ethanol and glucose were the biochemical experiments that were conducted.

Using fruit as a source of energy is a novel way to investigate renewable energy. This is because it has a high yield, is inexpensive, and contains sugar naturally without requiring a complicated hydrolysis pretreatment [10]. Soluble fermentable sugars including glucose, fructose, sucrose, cellulose, and hemicellulose structural fibres are present in the fruits. This fruit's biochemical makeup suggests that it may be a source of sugar that can be processed to produce ethanol.[11]. This data indicates that a wide variety of fruits can be utilised as bioethanol's raw materials. An easily accessible and eco-friendly chain for producing bioethanol from fruit without the need for hydrolysis or pretreatment is what makes this research unique.

The type of yeast is one of the variables that affects the amount of bioethanol produced. Therefore, in order to identify yeast isolates with the potential to produce bioethanol, yeast isolation and identification are required. The objective of this study is to isolate and characterise naturally occurring yeast from tropical fruits, evaluate the resistance of IY to glucose and ethanol, and assess its potential for in vitro bioethanol fermentation.

2. Method

The materials used in this research were local Indonesian fruit including jackfruit, Manalagi mango variety, pineapple and local grapes obtained from the market in Malang city. The media used for enrichment are YMM media (yeast maintenance media), distilled water, acetone, 70% alcohol, 0.05% chloramphenicol, methy lene blue coloring reagent, immersion oil, distilled water, cotton and spirit. Glucose tests and ethanol resistance tests were carried out on YMM media. Bioethanol production test using indigenous yeast in vitro on YMB (Yeast Malt Broth) media, *Saccharomyces cerevisae* isolate.

The tools used in this research were autoclave (DEA), vortex (Benchmark Scientific), laminar air flow (Innotech), analytical balance (Mettler), electric stove (Maspion), incubator (Memmert), cold centrifuge (Fisher Sci), shaker water bath (Memmert), Gas Chromatography (Shimadzu), microscope (Olympus), refrigerator, bunsen, tube needle, and glassware such as beakers, Erlenmeyer, measuring cups, measuring pipettes, test tubes and petri dishes.

2.1. Research Procedure

2.1.1. Isolation and Characterization of Indigeneous Yeast (IY). YMM media was made by weighing 6 g of yeast extract, 6 g of maltose, 10 g of peptone, 20 g of glucose then dissolving it in 2000 mL of

distilled water and heating until boiling while stirring until dissolved. Next, the media was put into two erlenmeyer 250 mL each and sterilized in an autoclave at a temperature of 121° C, pressure 15 psi for 15 minutes. The media was put into two Erlenmeyer flasks with a volume of 250 mL each, covered with cotton, and sterilized in an autoclave. YMB media: yeast extract 3 grams, Malt extract 3 grams, Peptone 5 grams, Dextrose 10 grams

Yeast isolation was obtained from several test fruits which were incubated in enrichment media for 72 hours (3 days). Yeast and Mold Medium (YMM) enrichment media consists of Yeast extract: 6 grams, Maltose: 6 grams, Peptone: 10 grams, Glucose: 20 grams and Aquadest: 2 liters. A total of 50 g of fruit was added to 450 ml of enrichment media.

2.1.2. Microscopic and Macroscopic Morphological of Yeast. Macroscopic observation techniques were carried out on yeast isolates resulting from 72 hour sub-culture which were placed on a glass deck dripped with sterile distilled water. Then it is observed under a microscope with a magnification of 4x10 to 100x10 by looking at the size, budding and shape of the cells. Macroscopic identification techniques include observing color, surface, texture, elevation and margins of yeast colonies [12].

2.1.3. Yeast Tolerance Test on Glucose. The yeast resistance test on glucose was carried out on YMM media at several glucose concentrations, namely 10%, 20%, 30%, 40% and 50%. Observations were made 48 hours after incubation with 3 replicates.

2.1.4. Yeast Tolerance Test on Ethanol. The yeast resistance test to ethanol was carried out on YMM media at several ethanol concentrations, namely 8%, 16% and 22%, observations were made 48 hours after incubation with 3 replicates.

2.1.5. Bioethanol Production Test. The bioethanol production test was carried out in vitro on YMB media. Take 100g of pineapple, grapes and jackfruit then crush them. Each fruit is then mixed with YMB media. The sample was added with 2 grams of *Sacharomyces cereviciae* as a control, and 2 grams of each yeast isolate with a fermentation time of 72 hours with 3 replicates. [13]. Bioethanol content measurements were carried out using GC-FID (*Gas Chromatography Flame Ionization Detector),* GC-agilent Technologies 6890-N Network GC System, HP InnoWax column 60 m long; diameter 0.25 µm with polyethylene glycol stationary phase, flame ionization detector (FID), carrier gas helium (He), and make-up nitrogen gas (auxiliary gas)

2.2. Data Analysis

The data analysis used is quantitative descriptive statistics. Data visualization is a form of descriptive statistical presentation that aims to present data in visual or graphic form so that it is more interesting and easier to understand. In this visualization, the data is presented in a variety of forms such as using tables, and bar diagrams (bar chart). Analysis data was carried out using Microsoft Excel 2010 software. The analysis technique of the yeast in each treatment and presented descriptively in the form of pictures and table. Yeast identify use referrences Kreger-van Rij, N. J. W. The Yeast: A Taxonomic Study. Amsterdam: Elsevier Science Publisher B. V. (1987). Yeasts: Characteristics and Identification 3rd Edition by J. A. Barnett, R. W. Payne, Cambridge University Press. 2021.

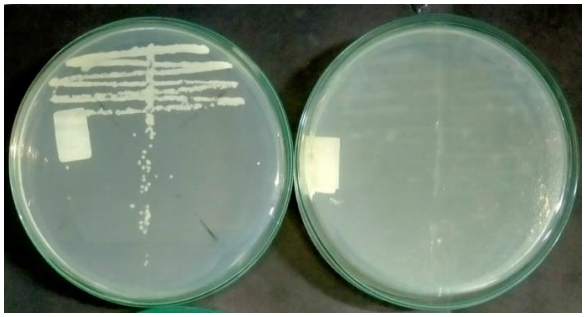
3. Results and Discussion

3.1. Isolation and characterization of indigenous yeast

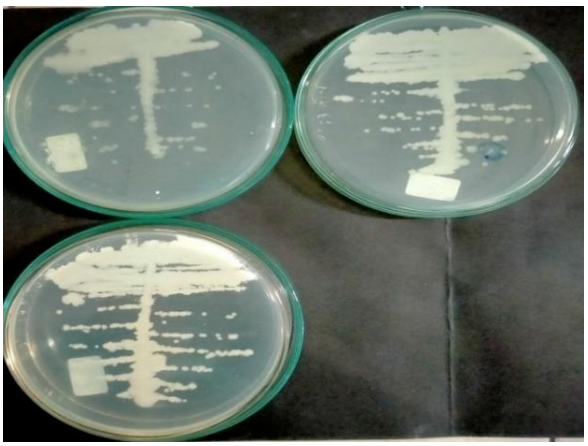
Isolation and characterization of yeast in grapes, pineapple, jackfruit and mango begins with ripening. Several types of fruit were marinated in YMM media for 3 days, then yeast was isolated. (Figure.1)

Figure 1. Fruit ripening on YMM media from left to right: grapes, mango, jackfruit and pinneapple

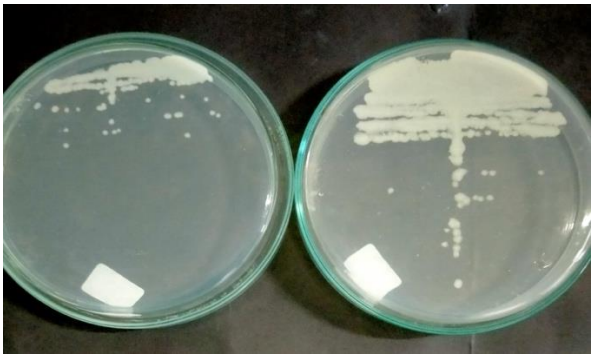
The results of yeast isolation in several types of fruit in enrichment media were then cultured in petri dishes. The culture results were observed for 1-3 days and on the media after yeast colonies appeared on each type of fruit. Yeast Sub Culture on YMM Media. The results of observations on yeast culture were obtained from 3 types of fruit that were successfully grown and then subcultured to grow yeast that was successfully isolated (Figure 2).



a. Isolates yeast from grapes



b. Isolates yeast from pineapple



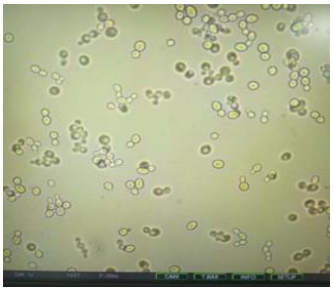
c. Isolates yeast from jackfruits

Figure 2. Morphology of subcultured yeast colonies from several fruits on YMM media for 48 hours incubation. a) grapes, b) pineapple, c). jackfruits

Taking naturally occurring microorganisms and cultivating them in a synthetic medium is the process of isolating and characterising them. The next stage involves identifying and separating the target microorganism from other microorganisms through a purification or separation procedure. Isolating a particular type of microbe from other microbes that originate from a mixture of different microbes is the fundamental idea behind microbial isolation. For the microbial cells to form a permanent cell colony, the microorganism needs to be growing in solid media. When Saccharomyces was isolated from grapes, it was discovered that 61 colonies with morphology similar to S. *cerevisiae* were the most dominant species [14].

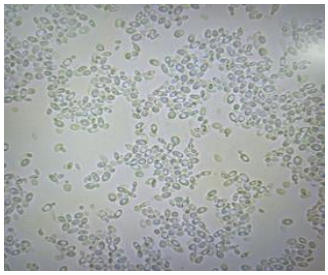
3.2. Microscopic of Yeast Morphology

Observation of yeast morphology was carried out after sub-culturing on YMM slant media using a microscope. The results of the observations showed that there were 7 types of yeast that were identified morphologically in Figure 3.

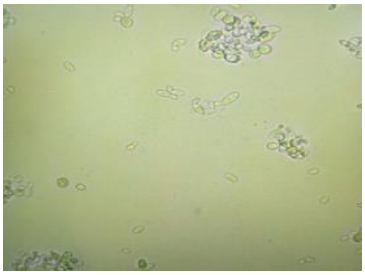


A1

A2



N1



N2

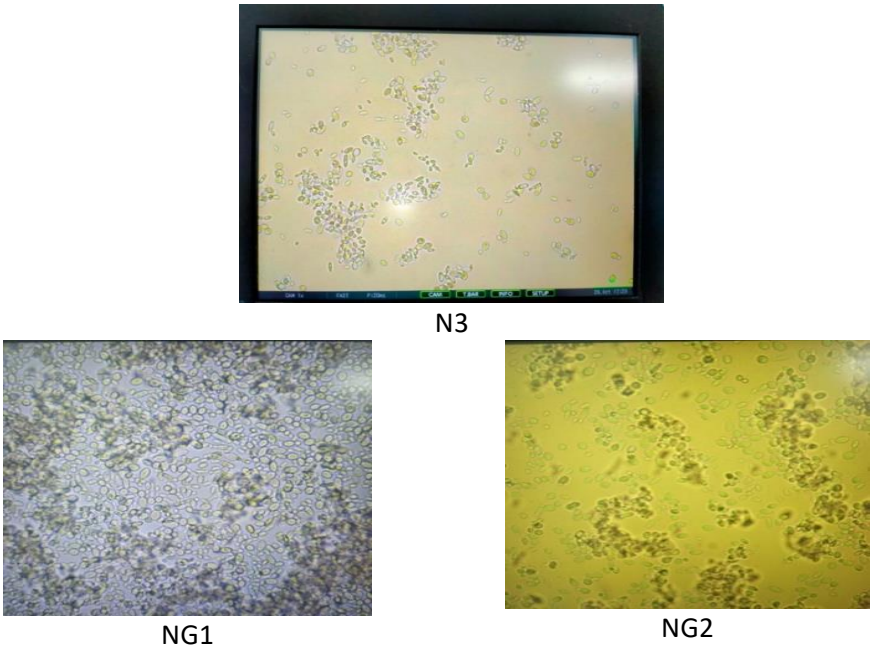


Figure 3. Observation of Yeast Morphology with a microscope with a magnification of 4x10 to 100x10, A1, A2 : grapes, N1,N2,N3 : pineapple, NG1,NG2 : jackfruits

Many kinds of yeast were isolated from each fruit. There were 2 types of yeast with codes A1 and A2 from grapes, and in pineapples there were 3 types of yeast with codes N1, N2 and N3 and in jackfruit, 2 types of yeast were found, namely NG1 and NG2. Yeast isolates were observed under a microscope, revealing asexual reproduction through budding. This observation led to the conclusion that the yeast isolates' asexual reproduction by budding placed them in the genus Candida, subclass Hemiascomycetes, and Saccharomyces. Asexual reproduction in the Hemiascomycetes subclass is accomplished by the formation of shoots, or budding [15]. [16]. The edges, elevation, colour, surface, texture, and shape of yeast isolates are among the microscopic morphological features that can be observed. The yeast isolates that were seen were growing independently as non-mucoid colonies. Another feature of yeast is that it is milky white or yellowish white in colour, has spores, lacks lustre, and smells like alcohol, according to the isolate's aroma.

3.3. Morphological Characteristics of Yeast

Using a microscope, one can observe the size, optics/color, shape, elevation, surface, and margins of yeast [6], [7]. Seven different types of yeast were identified from the subculture results, and their morphological traits were subsequently noted. There are three different character sizes: pinpoint, moderate, and small; colours: opaque, transparent, and translucent; shapes: circular and irregular; elevations: raised and flat; surfaces: smooth and shiny; margins and edges: entire, undulate, and lobate. (Table 1)

Table 1. Morphological Characteristics of Yeast

Code	Morphology description					
	Size	**Optic/Colour**	**Shape**	**Elevation**	**Surface**	**Margin**
A1	Pinpoint	Opaque	Circular	Flat	Shiny smooth	Entire
A2	Small	Transparent	Circular	Raised	Shiny smooth	Entire
A3	Moderate	Transparent	Irregular	Raised	Shiny smooth	Undulate
NG1	pinpoint	Translucent	Circular	Flat	Shiny smooth	Entire
NG2	moderate	Translucent	Circular	Flat	Shiny smooth	Entire
N1	pinpoint	Translucent	Circular	Flat	Shiny smooth	Undulate
N2	moderate	Opaque	Irregular	Raised	Shiny smooth	Lobate
N3	small	Translucent	Circular	Flat	Shiny smooth	Undulate

The morphological identification of yeast colonies and cells is done through the observation of both macroscopic and microscopic structures. We will compare the macroscopic and microscopic test results with yeast strains that are known to exist. If the goal is to determine the species level, these results are still weak. Based on the morphological resemblance of unknown isolates to known species, this morphological test can be used to estimate the genus of those isolates.

Based on microscopic and morphological observations, it was determined that the predominant colours of all yeast isolates were transparent, white, and cream. Because the yeast group Basidiomycetes contains carotenoids, their pigments are red, orange, yellow, and pink. Ascomycetes, on the other hand, lack colour pigments and are primarily white or cream in colour. The eight isolated yeast isolates were believed to belong to the Ascomycetes class due to their yellowish-white morphological appearance. [6]

The features of isolates with codes A1 and A2 are similar to those of the Candida sp genus. These include a circular colony shape, a smooth configuration, raised elevation, a clear white colour, and a shiny appearance. In the meantime, the cells have hyphae and pseudohyphae, and their morphology is oval-elongated/semi-spherical and cylindrical with a multilateral budding pattern. These traits align with the characteristics of Candida, which include round colonies, vegetative reproduction only (no sexual reproduction), white to cream colonies with buds. The genus Candida frequently produces hyphae or pseudohyphae. There is a lot of diversity in this genus of yeast. [15]

The NG1 and NG2 isolates share characteristics with the Saccharomyces genus, including a smooth/slippery configuration, a dull appearance, a convex elevation, a circular colony shape, and a clear white colour. The multilateral budding pattern and round-semi-round morphology of the cells are present. This genus can produce CO_2 and ethanol through anaerobic or semi-anaerobic fermentation on one or more forms of sugar. Thus, it can be said that the genus Saccharomyces contains this isolate. [7], [16].

3.4. Glucose Tolerance Test on Yeast

The yeast tolerance test on glucose was observed at 48 hours in Figure 4. The results of observations on glucose resistance showed that yeast was able to survive glucose up to a concentration of 8%. The types of yeast that are thought to be resistant to glucose are isolates N2 and NG 2.

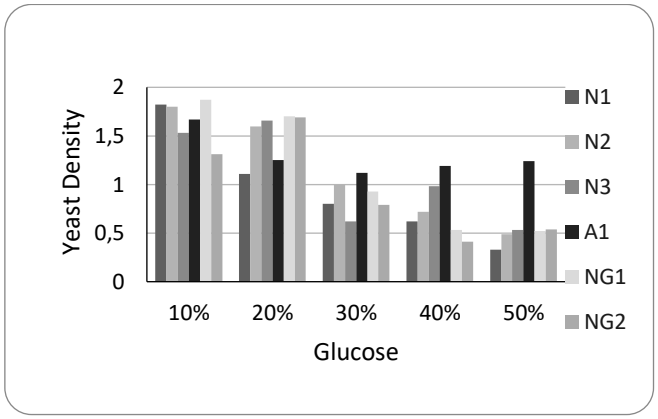


Figure 4. Yeast tolerance test on glucose at 48 hours observation

3.5. Ethanol Tolerance Test on Yeast

The yeast tolerance test on glucose was observed at 24 and 48 hours in Figure 5. The results of observations on ethanol resistance showed that yeast was able to survive on glucose up to a concentration of 8%. The types of yeast that are thought to be resistant to glucose are isolates N2 and NG 2.

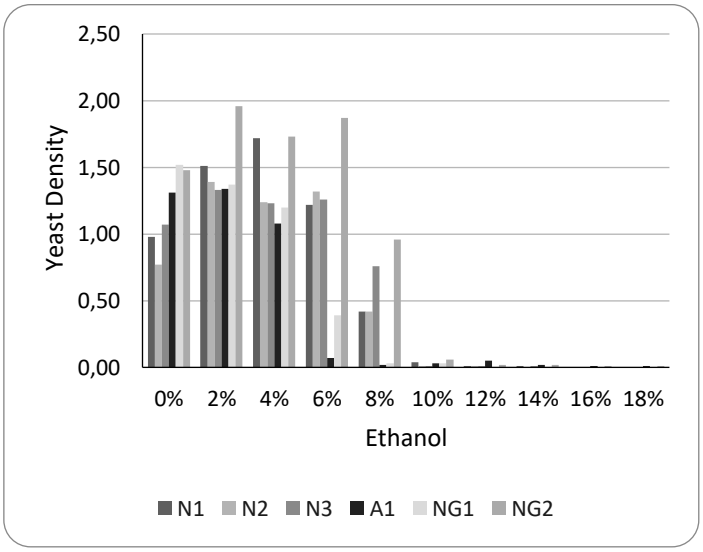


Figure 5. Yeast tolerance test on ethanol at 48 hours observation

In the ethanol tolerance test, the yeast's optical density value was determined using a UV-Vis Spectrophotometer with λ 600 nm. The number of cells growing in the test medium is indicated by the optical density value, which is directly related to the fermentation process that yeast cells carry out. 5% ethanol is the ethanol concentration that results in the highest optical density value of yeast isolates. This suggests that 5% is the ideal concentration for yeast isolates extracted from fruit. Yeast's optical density value drops as the concentration of ethanol increases. [6]

Because yeast cells are surrounded by cell walls made of mannoproteins and glucans, which can give yeast cells shape, yeast cells are able to withstand ethanol. In addition, cell walls shield the organism from the environment mechanically and thermally. Additionally, the cell wall can shield the cell membrane from turgor pressure, which could otherwise damage it. [17]

3.6. Bioethanol Production Test in Vitro

Testing of yeast's ability to produce bioethanol was carried out on YMB (Yeast Malt Broth) media. *S. cerevisae* was used as the control yeast in order to compare the yeast isolate's potential test. Using GC-FID, the amounts of bioethanol that each yeast isolate produced during fermentation was determined. The analysis's findings demonstrate that native yeast's capacity to produce bioethanol is still less than that of S. cerevisiae. One of the yeasts that is most frequently utilised in industrial fermentation processes is *S. cerevisiae*. Using effective yeast strains with high tolerance to ethanol will lower distillation costs, boost ethanol yield during fermentation, and benefit the environment. The ability of yeast to produce bioethanol is shown in Figure 6.

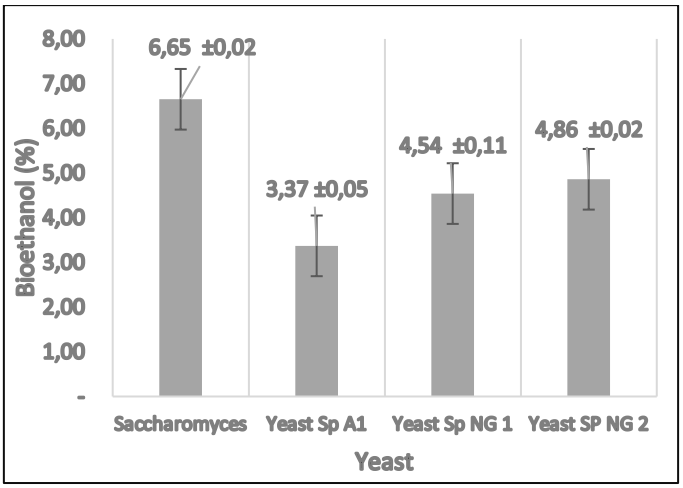


Figure 6. Potential yeast for bioethanol production (%)

There were only three isolates tested in the bioethanol production test: Yeast Sp A1, NG1, and NG2. This is the outcome of selection from earlier ethanol and glucose resistance tests. Sp NG2 yeast produced more bioethanol than other yeasts, but its results were still less than those of S. cerevisiae, according to the test results. According to the GC-FID test results, the fermentation of S. cerevisiae produced the highest bioethanol yield (6.65%), followed by the yeast isolate SpNG2 (4.86%). The observation results are described by the isolate code NG2 as having characteristics that are similar to those of the genus Saccharomyces because they have a smooth/slippery configuration, a circular colony shape, a clear white colour, a convex elevation, and flat edges (entire).and a dull appearance. Meanwhile, the cell morphology is round-semi-round with a multilateral budding pattern.

The lengthy fermentation treatment (12, 24, 36, 48, and 60 hours) had no discernible impact on alcohol content, according to the findings of a study on the effect of fermentation time on alcohol content in the bioethanol fermentation process from pineapple peel. After 48 hours of fermentation, 5.98 ± 1.01 g/L of bioethanol was produced from pineapple peel. Numerous investigations into bioethanol have been conducted in the past, with differing degrees of success. pineapple peel as a substrate, followed by four days of 24-33oC temperature fermentation with *S. cerevisae,* yielding an alcohol content of approximately 4.18-5.49%. [3]

Three days is the ideal amount of time for fermentation in the bioethanol production process. Fermentation that lasts longer than three days will result in less alcohol being produced. The reason for the lower alcohol content is that the alcohol has been changed into other substances, like esters. Variations in fermentation techniques lead to varying yields of bioethanol. Direct fermentation, independent hydrolysis and fermentation, and simultaneous saccharification and biomass fermentation are some of the techniques. This study employed direct fermentation as the method; alternative fermentation techniques were not compared. The study's findings indicate that glucose, uronic acid, xylose, galactose, arabinose, and mannose are the primary sugars that can be extracted from pineapple waste. The maximum ethanol yield of 3.9% (v/v) was attained during a concurrent 30-hour saccharification and fermentation period [13]. The SSF (Simultaneous Saccarification and

Fermentation) process is used to produce bioethanol from pineapple peel as a raw material. [18]. Squeezed pineapple juice contains 11% total sugar and 5% reducing sugar. The results show that optimal ethanol production is at a yeast content of 5% by weight and a fermentation time of 5 days resulting in ethanol production of 9.08g/l [19]. Bioethanol production from a mixture of banana, grape and mango peels shows that fruit peels containing sucrose increase alcohol production. The highest alcohol production was observed after 4 days (92 hours) in samples with sucrose and samples without sucrose [20]. The results of studies of yeast isolation from grape skins contained yeast that could be used in the fermentation process to produce ethanol [14]. The other study also showed that there is the yeast S. *cerevisiae* in pineapple fruit which can also produce bioethanol [21].

One of the crucial factors in determining how well yeast isolates are used is bioethanol. According to several studies, the majority of isolates generate bioethanol in the 7.1–12.0 percent range. A related study's findings indicated that it took 72 hours to reach the maximum ethanol concentration. Production of bioethanol steadily rose until the third day, at which point it fell. To generate relatively large amounts of ethanol, tolerance to higher sugar concentrations is required. In addition, another requirement for choosing yeast as a candidate for commercial bioethanol production is tolerance to ethanol. [22][21].

The degree of sugar utilisation during fermentation may be the cause of the variation in bioethanol production by various yeast isolates. The medium as suggested by the previously tested ethanol tolerance limits and reducing sugar concentration values. Many microbes have been used to ferment ethanol, but the most popular one is the yeast S. *cerevisiae,* which is currently the most commercial microorganism and grows perfectly at pH 4.8 and a temperature of ± 30^{o}C. [23].

The results of the bioethanol fermentation test using Saccharomyces yeast as a control showed that the bioethanol content in Saccharomyces: 6.60%, yeast species code N1: 3.30%, yeast species code NG1: 4.5%, yeast species code NG2: 4.85%. The morphology of isolates NG1 and NG2 has similar characters to the genus Saccharomyces because they have a circular colony shape, clear white colour, convex elevation, flat edges (entire), smooth/slippery configuration, and a dull appearance.

4. Conclusion

The results of isolation from yeast using selective media obtained 6 isolates with code A1 which was isolated from grapes, code NG1, NG2 which was isolated from jackfruit and codes N1, N2, N3 which were isolated from pineapple, while mango fruit was not found

Test results on glucose and ethanol resistance were selected for 3 isolates with codes N1, NG1 and NG2. The results of the bioethanol fermentation test using Saccharomyces yeast as a control showed that the bioethanol content in Saccharomyces: 6.60%, yeast species code N1: 3.30%, yeast species code NG1: 4.5%, yeast species code NG2: 4.85%

Acknowledgments

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