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# Screening Ethanol-Producing Yeast from Pineapple Honey *Ananas comosus* (L.) Merr var Quenn

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## Abstract

The availability of fossil-based fuels will gradually decrease while the amount required increases. This has prompted research to find alternative energy sources, including bioethanol. Bioethanol, as an alternative energy source, can be used as a transportation fuel better than fossil-based fuels because ethanol has a high octane number, laminar velocity and heat of vaporisation. Meanwhile, the level of carbon and hydrocarbon emissions is low so it can increase combustion efficiency in vehicles. In addition, the use of bioethanol as fuel is environmentally friendly and renewable. Ethanol can be produced through a fermentation process using ethanol-producing yeast. Pineapple honey is known for its high sugar and other nutrient content, so it is supposed that many types of yeast can grow well in it. This study aims to isolation and screening of bioethanol-producing yeast from Pineapple Honey. This study was carried out by yeast isolation method, primary yeast screening, secondary yeast screening, and characterisation of selected yeast isolates. Data on ethanol production by yeast isolates were analysed by ANOVA with $\alpha$=0.05 using the R studio program. This primary isolation and screening phase found eight yeast isolates capable of fermenting glucose and producing acid and gas in the test medium. Test ethanol production in DPE medium with 18% glucose content on all isolates showed that all isolates could produce ethanol with a range of 4.20-10.35%. Isolates of IK4 showed the highest ethanol production ability (10.35%) ($p<0.5\%$) in significant contrast to the other seven isolates. Based on macroscopic and microscopic morphological characterisations, IKM 4 isolates show similarities to the genus *Saccharomyces*.

**Keywords**: ethanol, pineapple, yeasts

## Introduction

Fossil fuel oil will gradually decrease in availability while its need increase (Shafiee and Topal, 2009). Bioethanol is a primary alcohol that is volatile, flammable, colourless, easily dissolved in water, and has a low boiling point of 78$^{o}$C. The characteristics of bioethanol make its function as an energy source more profitable than fossil fuels. In addition, the use of bioethanol as fuel is environmentally friendly and renewable.

Bioethanol can be produced sustainably by industrial fermentation processes. Bioethanol production by fermentation of raw sugar material requires resources from yeast as a microbial producer (Bušić et al., 2018).

Yeast is a microorganism of unicellular fungi that has a fermentative metabolic ability. Fermentation is carried out by yeast by breaking sugar into alcohol and gas. Yeast is found in places rich in sugar content, for example, in fruit. Fruits with high carbohydrate content include pineapple (Oguntibeju et al., 2013).

Pineapple fruit can be found in various regions in Indonesia (Hernosa et al., 2021). The storage period of Pineapple fruit is short because it is easily rotten by microbial activity. The nutritional content of pineapple, namely 17.53% carbohydrates and 13.65% reducing sugars, can be used as a source of growth for various types of yeast (Wijana et al., 1991; Chaudhary et al., 2019).

The high sugar content in Pineapple fruit can be a substrate for the growth of various wild yeasts. The diversity of yeast in natural substrates such as honey pineapple fruit has been isolated and tested for its potential to obtain superior ethanol producer yeast isolates.

## Materials and Methods

The materials used as a culture medium in this study were Potato Dextrose Agar (PDA) Medium, Yeast Malt Extract Agar (YMEA) Medium, 18% (w/v) Glucose Base Medium (GBM), Ethanol Producing Basic Medium (DPE), streptomycin antibacterial, peptone, and equates. The materials used for the observation of yeast characterisation

based on cell morphological properties are Sabaroud Agar Medium (SDA) for slide culture, Carrot Medium, Yeast Malt Broth Medium (YMB), Yeast Malt Agar Medium (YMA), and Glucose-Peptone-Yeast Extract Agar Medium (GPYEA). The dye solution used is Lactophenol and Methylene blue 0.01% (v/v), safranin, malachite green 0.5% (v/v) and basic fuchsin 0.05% (v/v). The pH indicator used is a bromothymol blue (BTB) solution.

The source of yeast isolate comes from the Honey Pineapple fruit, which consists of the flesh and skin of the fruit. The research design carried out includes four stages. The first stage is sample preparation and medium, followed by yeast isolation. The second stage is a primary screening of yeast isolates producing gas and organic acids when grown in glucose media. The third stage is a secondary screening of yeast isolates producing ethanol. The fourth stage is partial phenotypic identification of selected yeast isolate.

**Yeast Isolation from Isolate Sources**

The pureed Pineapple Honey sample is then serially diluted from 10-1 to 10-5. Each dilution is taken as much as 100 µL and inoculated with *the spread plate* technique on Petri dishes. Culture in PDA medium is then incubated for 2–5 days at 20–25ºC. The growing isolate is purified until a single colony is obtained that grows and is maintained in the YMEA medium. Purity of isolate culture is done by making colony-smeared preparations on the glass of the object and stained, then observed with a microscope.

**Yeast Primary Screening**

The yeast isolates were isolated after being subcultured for 48 hours in the YMEA medium, and then each was tested for growth on the GBM medium. The glucose fermentation ability of yeast is observed based on gas formation and changes in acid levels of culture media when yeast isolates were grown in a medium whose only carbon source is glucose. The test was repeated three times and incubated at 25ºC for 28 days.

**Yeast Secondary Screening**

Yeast isolates fermenting glucose was tested for their ability to produce ethanol. The yeast isolate was previously rejuvenated first. The yeast isolate was rejuvenated by culture for 24 hours in 18% (w/v) glucose GBM medium and incubated at 25ºC. Quantitative ethanol production tests were conducted by growing each yeast isolate rejuvenated in the DPE medium and statically cultured in the DPE medium at 25ºC for six days according to the Rosini *et al.* (1987) method with modifications.

Measuring ethanol levels produced by yeast uses an alcohol meter. Ethanol levels are calculated on fermented liquid that has been evaporated using a *rotary evaporator*. The heating temperature during evaporation is 65ºC for 15 minutes. Ethanol production is carried out by repeating three times. The difference in ethanol production for each yeast isolate was analysed by variety analysis (ANOVA), $\alpha$=0.05, using the R-4.3.1 for Windows program.

**Characterisation of Yeast Isolate**

A total of one of the highest ethanol-producing yeast isolates was identified based on vegetative and sexual cell morphological characterisations. Characteristics of vegetative reproduction are observed by the formation of buds, cell division, and conidia on short stems called sterigmata. Some yeast genera form asexual filaments and endospores (Hawksworth *et al*., 1983).

Colony and cell characteristics of vegetative yeast isolate were observed after yeast isolate was subcultured in liquid YM media. The morphology of yeast isolate colonies was observed in YM agar media and vegetative cells in culture slides grown for 1-7 days. Observed colony characteristics include texture, colour, surface, elevation, and margins. Vegetative cell morphology was also observed after cells were grown on YM liquid media for 2-3 days at 25ºC. Cells were observed with 0.01% *Lactophenol* and *Methylene blue* staining. Filamentation was observed in cultures grown in slides incubated for 21 days. The formation of asexual endospores was observed in old cultures on YM agar media at a temperature of 25ºC. The growing yeast isolates on carrot media observed bud structure, division, and conidia formation. Rejuvenated yeast isolates were then inoculated as much as one loop on carrot media and incubated ± for 7-21 days at 25ºC (Wickerham, 1951).

Characterisation of the sexual reproductive cell for the selected isolate was observed in ascus and ascospores. Description of ascospores was prepared by rejuvenating the isolate yeast culture first. The strain was rejuvenated by incubation in a cell culture tube containing YMEA agar media. A total

of one colony loop was inoculated on carrot medium and incubated ± for 7-21 days at 25ºC (Wickerham 1951). As many as one loops of the culture colony was made a thin smear on the glass of the object, then heat fixation and applied malachite green dye 0.5% and comparison dye safranin 0.5%. The preparation was observed in a microscope of 400x magnification (Kreger-van Rij, 1958).

## Results and Discussion

### Primary Screening of Gas-Producing Yeast in Glucose Fermentation

Isolation stage of yeast from honey pineapple Ananas comosus (L) Merr var Quenn obtained eight yeast isolates. This study showed that all yeast isolates during growth produce gas starting on the fourth day. In addition, all isolates caused a change in the pH of the culture to become acidic which was detected at the end of the observation (Table 1). According to Ratanapongleka et al. (2010), yeast is able to grow with glucose as the only carbohydrate source with indications of forming CO2 gas. In addition, yeast isolates that produce ethanol with a source of glucose will produce pyruvic acid, acetic acid, and carbon dioxide (Maicas, 2020).

Table 1. Changes in culture pH and gas formation during glucose fermentation by yeast isolate during the 7-day incubation

| Isolates | pH changes to acidic (+/-) | | | Gas bubble formation | | |
|---|---|---|---|---|---|---|
| | I | II | III | I | II | III |
| IKM 1 | + | + | + | + | + | + |
| IKM 2 | + | + | + | + | + | + |
| IKM 3 | + | + | + | + | + | + |
| IKM 4 | + | + | + | + | + | + |
| IKM 5 | + | + | + | + | + | + |
| IKM 6 | + | + | + | + | + | + |
| IKM 7 | + | + | + | + | + | + |
| IKM 8 | + | + | + | + | + | + |

### Secondary Screening of Ethanol-Producing Yeast

Secondary screening of yeast isolates as ethanol producers were carried out based on measuring ethanol levels on DPE media. Eight yeast isolates produced ethanol with ethanol levels ranging from 4.20-10.35% (v/v) (Figure 1). Statistical analysis of ethanol levels between isolates showed that IKM 4 yeast isolate was the highest ethanol-producing yeast strain (10.35% v/v) ($p<0.5$). The next high-production yeast isolates were IKM 7, IKM 8, IKM 3, IKM 2, and IKM 1. Between isolates IKM 5 and IKM 6 showed the lowest ethanol production (4.2% v/v) which was not significantly different ($p>0.5$).

Bioethanol production by IKM 4 isolate yeast was relatively high in the laboratory medium. The potential of this wild-type yeast isolate needs to be developed again using natural media that was cheaper and widely available in the environment and carried out by consortiums with other microorganisms. Bioethanol production by *Saccharomyces cerevisiae* species consortium with *Scheffersomyces stipitis* with rice straw substrate produces bioethanol with a concentration of 99% (Suriyachai et al., 2013). While *Saccharomyces cerevisiae* VSJ1. which was a consortium with *Aspergillus niger* (NCIM 1248) and damaged sorghum substrates produced bioethanol 2.90% v/v than damaged rice (2.09% v/v) (Suresh et al., 1999).

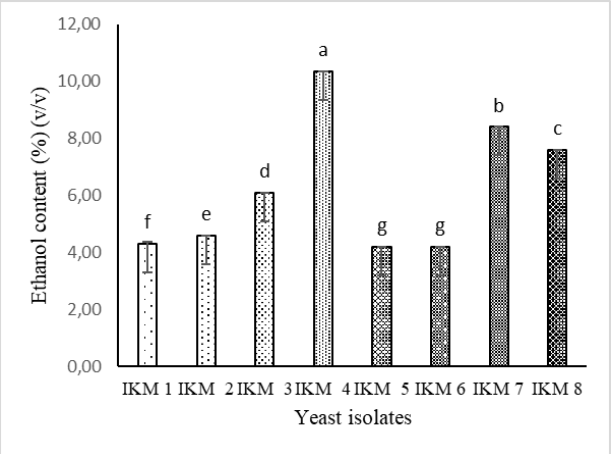


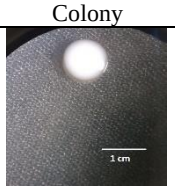
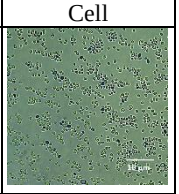
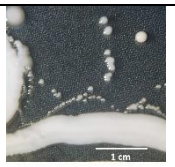
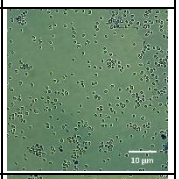
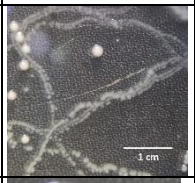
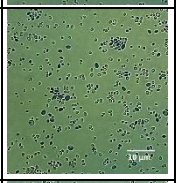
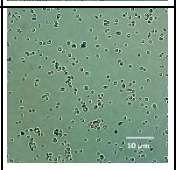
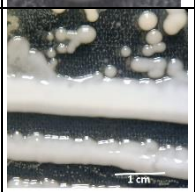
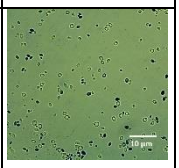
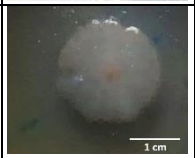
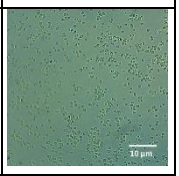
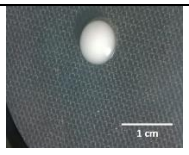
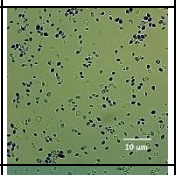
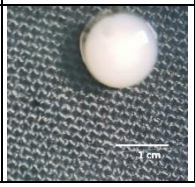
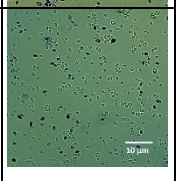

Figure 1. Production of ethanol by yeast isolates of honey pineapple (*Ananas comosus* (L) Merr var *Quenn*). Bars followed by different notations are significantly different ($p<0.05$).

### Characterisation of Selected Ethanol-Producing Yeast Isolate

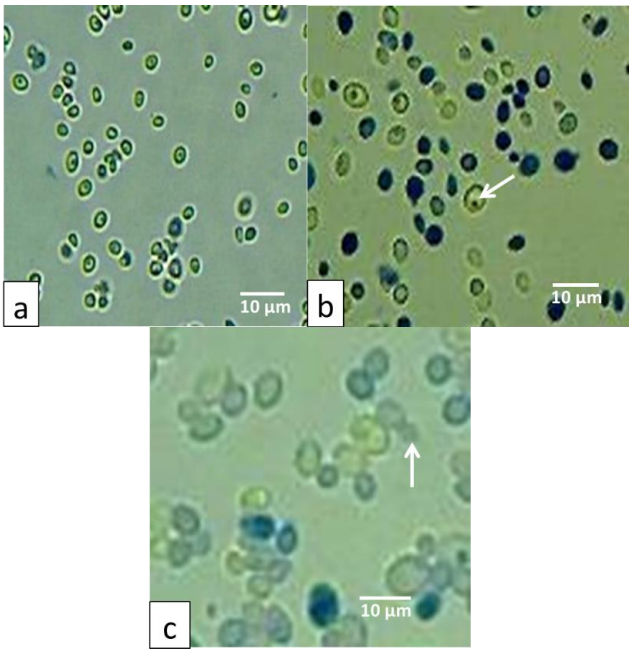
Eight yeast isolates showed macroscopic colony morphology similar, but microscopic cell morphology showed unique differences (Table 3). IKM 4 yeast isolate, a producer of ethanol levels with the highest average, has a round colony shape with flat colony edges. The colour of the upper and lower colonies is translucent white. The yeast colonies appear shiny with a slippery surface and convex elevation. Microscopically spherical cell shape, vegetative reproduction type forms budding or spherical budding, while ascospores are spherical in condition, with one ascus in the cell (Figure 2).

The morphological characteristics of the IKM 4 isolate have similarities with the genus *Saccharomyces* with differences in *pseudohyphae* formation.

Table 2. Morphology of yeast isolates of honey pineapple fruit *origin Ananas comosus* (L.) Merr var *Quenn*

| No | Isolate code | Macroscopic colony morphology | Microscopic cell morphology | Colony | Cell |
|---|---|---|---|---|---|
| 1. | IKM 1 | Colour: Clear white<br>Shape: round<br>Edge: flat<br>Surface: smooth and slippery<br>Appearance: shiny<br>Elevation: Convex | Oval cell shape |  |  |
| 2. | IKM 2 | Colour: Milky white<br>Shape: round<br>Edge: flat<br>Appearance: shiny<br>Surface: smooth<br>Elevation: Convex | Cell shape: cylindrical |  |  |
| 3. | IKM 3 | Colour: cloudy white<br>Shape: round Edge: flat<br>Appearance: shiny<br>Surface: smooth<br>Elevation:<br>Convex | Cell shape: oval |  |  |
| 4. | IKM 4 | Colour: Clear white<br>Shape: round (circular)<br>Edge: flat<br>Appearance: shiny<br>Surface: smooth and slippery<br>Elevation: convex | Cell shape: round |  |  |
| 5. | IKM 5 | Colour: The cloudy creamy white<br>Shape: round<br>Edge: flat<br>Appearance: cloudy<br>Surface: smooth and slippery<br>Elevation: Convex | Cell shape: cylindrical |  |  |
| 6. | IKM 6 | Colour: cloudy white<br>Shape: round<br>Edge: flat<br>Appearance: cloudy<br>Surface: smooth and slippery<br>Elevation: Convex | Cell shape: round |  |  |
| 7. | IKM 7 | Colour: precise white Shape: round<br>Edge: flat<br>Appearance: shiny<br>Surface: smooth and slippery<br>Elevation: Convex | Cell shape: oval |  |  |
| 8. | IKM 8 | Colour: Clear white<br>Shape: round<br>Edge: flat<br>Appearance: shiny<br>Surface: smooth and slippery<br>Elevation: convex | Cell shape: cylindrical |  |  |

This study found eight yeast isolates from pineapple honey that produce ethanol (4.2-10.35%). IKM 4 yeast isolate produced the highest ethanol with a content of 10.35%, while IKM 5 and IKM 6 yeast isolates produced ethanol with the lowest range of 4.20%. Partial identification based on morphological characterisation of colonies and vegetative and generative cells showed that IKM 4 yeast isolates were similar to the genus *Saccharomyces*.




(a) round cell shape; (b) ascospore; (c) budding cell

Figure 2. Microscopic characteristics of a yeast isolate IKM 4

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