ETHANOL FERMENTATION POTENCY OF WILD YEAST ON BAMBOO RHIZOSPHERE

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ABSTRACT

Bamboo plant rhizosphere known as source of simbiotic useful microorganisms, including yeast. Wild yeast explored should be tested it’s adaptability to new ecology especially nutritional source availability. The research aim to get potential yeast which can work well during fermentation process in apple juice substrate.

We were isolated yeast from three different locations: Ketawanggede District, Karangploso District, and Lowokwaru District. All locations was located around Malang city.

Result showed that there were found 13 isolates yeast: Protomyces sp, Agaricostilbum sp1, Agaricostilbum sp2, Agaricostilbum sp3, Debaryomyces sp1, Debaryomyces sp2, Debaryomyces sp3, Trigonopsis sp1, Trigonopsis sp2, Udeniomyces sp1, Udeniomyces sp2, Ascoidea hylocieti, and Komagataella sp. Diversity index indicates medium category to low category and dominance index in all location indicates high category.

Fermentation test showed improvements in observation variables at 24 and 72 hours including temperature, cells number, and alcohol percentage. The highest alcohol percentage were 11.6% and 10% that produced by the treatment of Agaricostilbum sp3 and Trigonopsis sp1 respectively.

Keyword: Yeast, bamboo plant rhizosphere, apple juice, and fermentation.

While oxidative (respiration) can produce carbon dioxide and water (Fardiaiz, S., 1992). Some yeast belonging to the fermentative yeast include Saccharomyces, Candida, Brettanomyces and Zygosaccharomyces. While non fermentative yeast is Rhodotorula (Van Dijken, J.P. and W. A. Scheffers, 1986).

Yeast has been used for industrial processes such as the manufacture of alcoholic beverages, fermented tape, the manufacture of animal feed, cosmetics, and antibiotics (Tanaka, et., al., 1990; Ardhana, M.M. and G.H. Fleet, 1989). Yeast in the future can be developed as renewable resources, because some types of yeast capable of producing alcohol from a variety of different types of carbohydrates. Various exploration will find yeasts especially the types of yeast that have potential in the fields of industry, particularly in the production of bioethanol (Lansane, B.K., G. Vijayalakshi, M.M. Krishnaiah, 1997).

Research on yeast is mostly done in exploration of various ecosystems in Indonesia. It is believed that the amount of yeast in nature is much higher than yeast that has been known for. Research has been done that yeast exploration conducted on the district of Jember, East Java Some yeast isolates were obtained among other Candida sp. 1, Candida sp. 2, Candida sp. 3, Debaryomyces sp. and Kloeckera sp. (Muhibuddin, A and I.R. Sastrahidayat, 2015; Ivanesthi, IR., S. Nurhatika, dan A.Muhibuddin, 2016).

The purpose of this research was to observe the potential of wild yeast from bamboo ethanol fermentation, because it’s potential (Singhal, P., 2013)

2. METHODS

2.1. Time and Place

Research was conducted at the Mycology Laboratory, Faculty of Agriculture, University of Brawijaya. Research has been conducted
from February till June 2016. Soil samples obtained from bamboo plant roots in three locations: Ketawanggede District, Karangploso District, and Lowokwaru District.

2.2. Materials

The laboratory equipments were used in the research consisted of plastic trays, Petridishes, bottles Duran Schott, measuring glass Pyrex, Erlenmeyer flask Duran Schott, beaker glass Duran Schott, object glass and cover glass Sail Brand Microscope slides 23 Cat No. 7101, Pasteur pipette, micropipette Vit Lab 100 mL, ose needles, scales Ohaus Gent-0-gram balance 311 g, test tubes, spatulas, bunsen, microscope camera Olympus BX 41+ OP 26, handsprayer, autoclave Hirayama, orbital shaker Protech, laminar Air Flow Cabinet (LAFC), UV-VIS spectrophotometer, Spetkoquant Pharo 300, pH meter T1 Trans Instruments Lab 900 Walk a microcomputer technology, and bottle fermentation.

Materials used in the research include YMA media (Yeast Malt Agar), YMB media (Yeast Malt Broth), SB media (Saboroud Broth), alcohol 70%, NaOCl, distilled sterile water, spirits, matches, composite soil samples, (NH$_4$)$_2$SO$_4$, H$_2$SO$_4$, and apples.

2.3. Research Design

The research was conducted using 14 treatments and each treatment was repeated three times.

The treatments were:

P$_0$ : Control
P$_1$ : Protomyces sp
P$_2$ : Agaricostilbum sp1
P$_3$ : Debaryomyces sp1
P$_4$ : Trigonopsis sp1
P$_5$ : Debaryomyces sp2
P$_6$ : Udeniomyces sp1
P$_7$ : Ascoidea hylocieti
P$_8$ : Agaricostilbum sp3
P$_9$ : Komagataella sp
P$_{10}$ : Udeniomyces sp2
P$_{11}$ : Agaricostilbum sp3
P$_{12}$ : Trigonopsis sp2
P$_{13}$ : Debaryomyces sp3

2.4. Soil Samples Exploration

Samples isolated by collect composites soil samples from the bamboo plant rhizosphere. Soil sample was taken at 15 cm depth (Ashliha et al., 2014).

2.5. Yeast Isolation and Purification

10 grams soil sample put to the Erlenmeyer that contained 90 ml of distilled water. The mixture then homogenized and deposited ± 5 minutes. Supernatant was taken at 10 ml and put in a flask contained 40 ml of media YMB. Then incubated on top orbital shaker at room temperature for three days. For dilution taken 1 ml suspension from the Erlenmeyer contained isolates from YMB, then put into a test tube that contained 9 ml sterile distilled water and gained up to $10^5$ serial dilution. After that, taken 0.1 ml suspension and inoculated on YMA media with spread methods. Yeast grown on YMA media incubated at room temperature for about 3 days and purification to obtained pure colonies. Observations were made on the characteristics of the macroscopic colonies grown on media (Ashliha et al., 2014).

2.6. Yeast Identification

Yeast isolates were identified to the genus by referring to the identification guide book "The Yeast a Taxonomic Study". Observations were made macroscopically and microscopically. Macroscopic observation was based on the appearance of colony morphology upon isolation and purification include the shape, texture, color, surface, elevation, and the waterfront. Microscopic observations on yeast include cell shape, size, type of budding, presence or absence of hyphae or pseudohyphae and spore types were obtained from isolates (Widiastuti et al., 2014). Microscopic observation use preparation cultured on glass objects and characters seen in microscope (Ashliha et al., 2014).

2.7. Yeast Growth

Yeast growth in liquid media test to know the mechanism of carbohydrate utilization by the yeast. One ose colonies of YMA was added to test tubes that contained 10 ml SB media and grown for 24 hours. Yeast oxidative in SB media will form a layer or pellicle on the surface of the media, while the
fermentative yeast will form a layer or pellicle on the basis of media (Jumiyati et al., 2012).

2.8. Substrate Fermentation

5 kg apple was washed with tap water. Then the apples crushed using a blender with add water ratio 2:1 (w/v). and then squeezed to separate the fiber and fruit extract. Pasteurization apple juice at temperature of 65-70° C for 15-20 minutes. Then added 1% (NH₄)₂SO₄ (v/w) and 10% sugar (v/w) to the apple juice. Then added H₂SO₄ or NaOH in apple juice to adjust the pH to the range 4.5-5 for optimum growth of yeast (Sigit et al., 2014).

2.9. Yeast Activators

Apple juice prepared in the Erlenmeyer flask for diluted up to 100 ml. Then added 1% (NH₄)₂SO₄ (v/w) in apple juice. Apple juice heated up to boiling and cooled in a state phase. Then added H₂SO₄ or NaOH in apple juice to adjust the pH optimum conditions growth of yeast. Add 20 ml the apple juice solution to the Erlenmeyer. After that, added one ose yeast isolates that taken from propagation on YMA media in apple juice solution and incubated on an orbital shaker for 24 hours (Santi, 2008).

2.10. Fermentation Test

100 ml fermentation substrate was added to the bottle fermentation. After that, added 6% yeast activator solution. Then covered the bottle fermentation with aluminum foil to exclude the contamination by other microbes. The fermentation process was conducted for 72 hours (Sigit et al., 2014).

2.11. Variable Observations

The exploration data was analysed by Diversity Index (H’), Evenness Index (E), and the Dominance Index (C) with each Ludwig et al. (1998) in Sidiyasa et al. (2006) as follows:

\[ H' = \Sigma (pi \ln pi) ; \pi = ni/N \quad \text{(1)} \]

- **H’** = Shannon Diversity Index
- **ni** = Number of individuals
- **N** = Number of total individuals of all species
- **Pi** = The proportion of an individual species in relation to all types

\[ E = \Sigma H'/\ln(S) \quad \text{(2)} \]

- **E** = Evenness Index Pielou
- **Ln** = logarithm normal
- **S** = Amount of types

\[ C = (ni/N)^2 \quad \text{(3)} \]

- **C** = Dominance Index
- **ni** = Amount of individuals in a type
- **N** = Amount of individuals all types

Observation of physical characteristics such as color observed by observe color changes during fermentation. Physical characteristics such as smell was observed during fermentation. Both above characteristics were observed every 24 hours. Observation cells numbers was observed every 24 hours. The number was calculated using a UV-VIS spectrophotometer with a wavelength of 600 nm (turbidimetry methods). pH and physical characteristics such as temperature was observed using T1 900.

The ethanol percentage was measured using picnometer and rotary evaporator. The rotary evaporator was used to gain the ethanol solution from the fermentation medium, while the picnometer was used to measure the ethanol value once evaporation was performed. The ethanol value measurement was done by obtaining the specific weight with picnometer. First, the picnometer was filled with aquades, then both were sealed and weighted, resulting in W2; the picnometer was thereafter emptied, the remaining aquades absorbed with acetone. The picnometer was dried in an oven and thereafter weighted for the second time, resulting in W1. The weight of aquadest (W) was obtained by subtracting W2 to W1.
2.12. Data Analysis

Quantitative data analyzed descriptively using charts. The qualitative data analyzed descriptively.

3. RESULT AND DISCUSSION

3.1. Yeast Exploration

Yeast that were isolated: Protomyces sp (1), Agaricostilbum sp1 (2), and Debaryomyces sp1 (5). From Karangploso area soil sampling include Agaricostilbum sp2 (3), Debaryomyces sp2 (6), Trigonopsis sp1 (10), Udeniomyces sp1 (8), Ascoidea hylocieti (12), and Komagataella sp (13). From Lowokwaru area soil sampling include Agaricostilbum sp3 (4), Debaryomyces sp3 (7), Trigonopsis sp2 (11), and Udeniomyces sp2 (9).

Table 1. Diversity Index

| Location       | Index | Category |
|----------------|-------|----------|
| Ketawanggede   | 1.06  | Medium   |
| Karangploso    | 0.97  | Low      |
| Lowokwaru      | 1.08  | Medium   |

Table 2. Evennes Index

| Location       | Index | Category |
|----------------|-------|----------|
| Ketawanggede   | 0     | Low      |
| Karangploso    | 1     | High     |
| Lowokwaru      | 1     | High     |

Table 3. Dominance index

| Location       | Index | Category |
|----------------|-------|----------|
| Ketawanggede   | 0.33  | High     |
| Karangploso    | 0.4   | High     |
| Lowokwaru      | 0.33  | High     |

Table 4. Macroscopic Characteristics of Yeast

| Isolate | Elevation  | Colour     |
|---------|------------|------------|
| 1       | Little convex | Ivory     |
| 2       | Little convex | White like candle |
| 3       | Little convex | White like candle |
| 4       | Little convex | White like candle |
| 5       | Little convex | White yellowish |
| 6       | Little convex | White yellowish |
| 7       | Little convex | White yellowish |
| 8       | Flat- little convex | White like candle ivorys |
| 9       | Flat- little convex | White ivorysh |
| 10      | Flat        | White like candle |
| 11      | Flat        | White like candle |
| 12      | Flat- little convex | Egg white |
| 13      | Flat        | White like candle |

Table 5. Microscopic Characteristic of Yeast

| Isolate Code | Size of Cell | Type of Bud | Shape of Cell |
|--------------|--------------|-------------|---------------|
| 1            | 1,47-4.14 μm | Multi-polar | Spheroidal    |
| 2            | 1,12-3.4 μm | Monopolar   | Spheroidal    |
| 3            | 1,16-3.42 μm | Monopolar   | Spheroidal    |
| 4            | 1,16-3.42 μm | Monopolar   | Spheroidal    |
| 5            | 1,06-4.42 μm | Monopolar   | Spheroidal    |
| 6            | 1,09-4.42 μm | Monopolar   | Spheroidal    |
| 7            | 1,09-4.42 μm | Monopolar   | Spheroidal    |
| 8            | 1,05-1.54 μm | Monopolar   | Spheroidal    |
| 9            | 1,05-1.53 μm | Monopolar   | Spheroidal    |
| 10           | (1.05 x 1.95) - (1.07 x 2.85) μm | Multi-polar | Triangular or cylindrical |
| 11           | (1.05 x 1.95) - (1.07 x 2.85) μm | Multi-polar | Triangular or cylindrical |
| 12           | 1,04-6.85 μm | Multi-polar | Spheroidal    |
| 13           | 1,04-6.85 μm | Multi-polar | Spheroidal    |

Table 6. Yeast Physiological Characteristic

| Isolate Code | Physiological Characteristic |
|--------------|-----------------------------|
| P0           | Obligate fermentative       |
| P1           | Facultative fermentative    |
| P2           | Facultative fermentative    |
| P3           | Facultative fermentative    |
| P4           | Facultative fermentative    |
| P5           | Obligate fermentative       |
| P6           | Facultative fermentative    |
| P7           | Facultative fermentative    |
| P8           | Facultative fermentative    |
| P9           | Facultative fermentative    |
| P10          | Oksidative                  |
| P11          | Obligate fermentative       |
| P12          | Facultative fermentative    |

3.2. Fermentation Test

1. Alcohol content

At 72 hour observation showed that the control treatment had the lowest alcohol level (1.3%). Fermentation test results showed that the highest alcohol level of 11.6% produced by
Agaricostilbum sp3. Other results showed that the alcohol level of 10% was produced by Trigonopsis sp1, 9.3% by Debaryomyces sp1, 9% by Agaricostilbum sp1, 7.4% by Debaryomyces sp2, 6.8% by Debaryomyces sp3, 5.6% by Udeniomyces sp2, 5.4% by Trigonopsis sp2, 5% by Agaricostilbum sp2, and 4.6% by Ascoidea hylocieti and Protomyces sp.

Alcohol Content (%)

| Yeast Isolates | Alcohol Content (%) |
|----------------|---------------------|
| Control        | 1.3                 |
| Protomyces sp  | 9                   |
| Agaricostilbum | 9.3                 |
| Debaryomyces sp1 | 10                |
| Udeniomyces sp1 | 7.6                 |
| Ascoidea       | 4.6                 |
| Agaricostilbum | 5                   |
| Debaryomyces sp2 | 5.6                |
| Udeniomyces sp2 | 5.4                 |
| Komagataella sp | 6.8                 |

Then, 4% alcohol content from Komagataella sp., and 3.1% from Udeniomyces sp1 treatments respectively.

3. Temperature

The fermentation process begins with temperature increase, and after the highest point the temperature will decrease to approximately the same temperature as the beginning of the fermentation process. Test fermented for 72 hours were performed using apple juice show the temperature increasing and decreasing, although still in the range of 26-27°C.

Temperature fluctuations is not significant as long as possible because the fermentation bottle cap was opened every 24 hours for measurement of temperature and pH. It indirectly provides different treatment for their mixing process when the electrode was inserted. The measuring process indirectly affect the discharge of carbon dioxide is a byproduct of the fermentation process. Carbon dioxide was formed by the natural fermentation process can affect the temperature during the fermentation process. The loss of carbon dioxide indirectly affect to temperature. Yeast can produce alcohol in an optimal temperature range of 30-33°C (Azizah et al., 2012).

4. Smell

Fermentation test was conducted for 72 hours showed a change its smell when compared to observation 0 hours. The changes generally occurs on all substrates with each treatment and the addition of 13 yeast isolates tested. Control unchanged smell due to the absence of additional yeast isolates. The changes as well be an indication of the formation of alcohol during the fermentation process.

5. Color

Substrate fermentation test that using a solution of apple cider shows fawn at 0 hours of observation on the overall treatment with the addition of yeast isolates. Control treatment was a different color than the other treatments on observations 0 hours because she did not isolate the addition of yeast. Colors were observed at 0 hour show differences in observation 72 hours, so that indicates a change in nutrition during fermentation test. Azizah et al. (2012) mentions that the fermentation substrate will change...
physically because of the overhaul of complex compounds into compounds simpler.

Control treatment does not change the color so that the color remains dark brown in 72 hours of observation. Test fermentation with the addition of isolates Protomyces sp, Agaricostilbum sp1, Debaryomyces sp1, Trigonopsis sp1, Debaryomyces sp2, Ascoidea hylolociet, Agaricostilbum sp2, Komagataella sp, Agaricostilbum sp3, Trigonopsis sp2, and Debaryomyces sp3 shows a color change from yellowish brown to yellow-brown on observations of 72 hour. Addition isolates of Udeniomyces sp1 and Udeniomyces sp2 shows similarities with the control treatment that is not a color change on the observation 72 hours with fixed color is yellowish brown.

6. Optical Density

Observations commonly show OD values indicate that the observation 0 hours until 72 hours of observation yeast still in the range of numbers 1, except for the treatment with the addition that the isolates Agaricostilbum sp1 observation 72 hours reached a value of 2. The increase in OD up to 72 hours of observation on the all treatment added of activators showed that the alcohol level produced during fermentation test can still be tolerated by the yeast, so the yeast is still the potential to produce higher alcohol because yeast can still breed. Azizah et al. (2012) states that a certain level of alcohol can inhibit the growth of yeast.

OD values among treatment showed no significant difference. The results of cell counting using turbidometry method is strongly influenced by the cell size and color density of a test solution.

7. Relations Between Media Growth and Fermentation Result

Protomyces sp has obligate fermentative characteristics and the resulting alcohol level is 4.6%. Agaricostilbum sp1 has facultative fermentative characteristics and the resulting alcohol level of 9%. Debaryomyces sp1 has facultative fermentative characteristics and the resulting alcohol level is 9.3%. Trigonopsis sp1 has facultative fermentative characteristics, as well as the resulting alcohol is 10%. Debaryomyces sp2 has facultative fermentative characteristics and the resulting alcohol level is 7.4%. Udeniomyces sp1 has facultative fermentative characteristics and the resulting alcohol level is 3.1%. Ascoidea hylolociet have facultative fermentative characteristics and the resulting alcohol level is 4.6%. Agaricostilbum sp2 has a facultative fermentative characteristics and the resulting alcohol level of 5%. Komagataella sp has a facultative fermentative characteristics and the resulting alcohol level is 4%. Udeniomyces sp2 have a facultative fermentative characteristics as well as the resulting alcohol level of 5%. Agaricostilbum sp3 has a facultative fermentative characteristics and the resulting alcohol level is 11.6%. Trigonopsis sp2 has obligate fermentative characteristics and the resulting alcohol level is 5.4%. Debaryomyces sp3 has a facultative fermentative characteristics and the resulting alcohol level was 6.8%.

The highest of alcohol produced from the fermentation test with the addition of Agaricostilbum sp3 isolates that have oxidative characteristics. This can occur because unscrew bottle fermentation process when observation of pH and temperature. Bottle fermentation which opened indirectly remove carbon dioxide contained in the bottle fermentation, and causing the entry of oxygen into the bottle fermentation. Excessive carbon dioxide in the bottle fermentation can inhibit the growth of yeast. In addition, the alcohol level produced by yeast metabolism will be more on aerobic conditions that do require oxygen. The conditions according to the oxidative properties of yeast, so that the resulting alcohol level higher than treatment with the addition of other isolates.

4. CONCLUSION

1. We found 13 yeast isolates from 3 locations: Protomyces sp, Agaricostilbum sp1, Agaricostilbum sp2, Agaricostilbum sp3, Debaryomyces sp1, Debaryomyces sp2, Debaryomyces sp3, Trigonopsis sp1, Trigonopsis sp2, Udeniomyces sp1, Udeniomyces sp2, Ascoidea hylolociet, and Komagataella sp. that all isolates are fermentative yeast, except Agaricostilbum sp3.

2. The highest alcohol percentage was on treatment Agaricostilbum sp3.
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