ETHANOL FERMENTATION POTENCY OF WILD YEAST ON BAMBOO RHYZOSPHERE

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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.

1. INTRODUCTION

Yeast is one of microorganisms belong to unicellular fungi. Based on the type of yeast metabolism, yeast can be divided into two groups: fermentative yeast and oxidative yeast. Fermentative types can perform alcoholic fermentation, which breaks down sugar (glucose) into alcohol, lactic acid and gas. While oxidative (respiration) can produce carbon dioxide and water (Fardiaz, 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, Spektroquant 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, $(NH_4)_2SO_4$, H_2SO_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 (Widiastutik *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 65-70º C for 15-20 minutes. Then added 1% $(NH_4)_2SO_4$ (v/w) and 10% sugar (v/w) to the apple juice. Then added H_2SO_4 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% $(NH_4)_2SO_4$ (v/w) in apple juice. Apple juice heated up to boiling and cooled in a state phase. Then added H_2SO_4 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:

$$(H') = \Sigma (pi \ln pi) \; ; pi = ni/N \quad \ldots\ldots\ldots (1)$$

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

$$E = \Sigma H'/\ln(S) \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

H' = Diversity index
E = Evenness Index Pielou
Ln = logarithm normal
S = Amount of types

$$(C) = \Sigma (ni/N)^2 \quad \ldots\ldots\ldots\ldots\ldots\ldots (3)$$

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

Exploration data based on Jukri *et al*. (2014) categories:

Diversity index	High	< 3
	Medium	1-3
	Low	> 1
Evennes index	High	0
	Low	1
Dominance index	Hight	0
	Low	1

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 substracting 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	Mono-polar	Spheroidal
3	1,16-3,42 µm	Mono-polar	Spheroidal
4	1,16-3,42 µm	Mono-polar	Spheroidal
5	1,06-4,42 µm	Mono-polar	Spheroidal
6	1,09-4,42 µm	Mono-polar	Spheroidal
7	1,09-4,42 µm	Mono-polar	Spheroidal
8	1,05-1,54 µm	Mono-polar	Spheroidal
9	1,05-1,53 µm	Mono-polar	Spheroidal
10	(1,05 x 1,95) - (1,07 x 2,85) µm	Multi-polar	Triangular or cilyndrical
11	(1,05 x 1,95) - (1,07 x 2,85) µm	Multi-polar	Triangular or cilyndrical
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
P_0	Obligate fermentative
P_1	Facultative fermentative
P_2	Facultative fermentive
P_3	Facultative fermentative
P_4	Facultative fermentative
P_5	Obligate fermentative
P_6	Facultative fermentative
P_7	Facultative fermentative
P_8	Facultative fermentative
P_9	Facultative fermentative
P_{10}	Oksidative
P_{11}	Obligate fermentative
P_{12}	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.

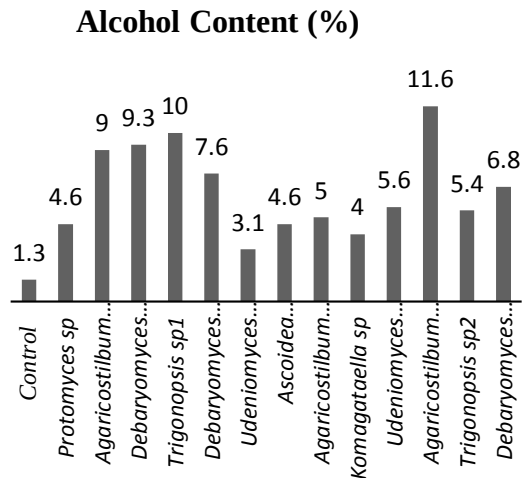

Figure 1. Alcohol Content After 72 hours Fermentation

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

2. pH

Decreasing of pH from all treatments indicates that there are fermentation process on apple juice. The highest decrease at observation between 0 hours to 24 hours was using *Agaricostilbum* sp1.

The highest decreasing of pH between observation at 24 hours to 48 hours was using *Komagataella* sp. isolates with a pH of 4.48 to 3.49.

The highest decrease in pH between observation at 48 hours to 72 hours in the fermentation treatment with added activators of *Debaryomyces* sp2 with pH 3.93 to 3.68 on a 24-hour observation.

Decrease in pH is generally influenced fermentation process by the yeast that produces a byproduct such as alcohol and carbondioxide that acidic chracter (Kartohardjono 2007 in Azizah *et al*.,2012).

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 26-27ºC.

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 carbondioxide is a byproduct of the fermentation process. Carbondioxide was formed by the natural fermentation process can affect the temperature during the fermentation process. The loss of carbondioxide indirectly affect to temperatur.Yeast can produce alcohol in an optimal temperature range of 30-33ºC (Azizah *et al*., 2012).

4. Smell

Fermentation test was conducted for 72 hours showed a change it's 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 hylocieti*, *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 turbidimetry 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 obligate 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 hylocieti* 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%. *Udenimyces* 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 hylecoeti*, 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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