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Ethanol Fermentation Potency of Wild Yeast Which Isolated from Soil Drive Nutrient (SDN) Plantation System

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ABSTRACT

Yeast which has a fermentative type can ferment alcohol, which break down the sugars into alcohol, lactic acid and gas. This yeast's type is needed in biofertilizer process production. The purpose of this study was to observed the potential in ethanol production of the soil wild yeast that isolated from Jember, East Java and determine the concentration of sugar product as well as the best time of ethanol fermentation. Our previously observation have found 113 yeast species. Then we select them based on fermentative ability and found five best species. These five species then have determined its sugar product as well as the best time of ethanol fermentation and choose two best. We have counted the level of ethanol production using glucose medium of 10%, 15% and 20% of these two best species.

The results showed that *Candida* sp. 1 (species number-1) and *Candida* sp. 3 (species number-3) has a better potential to produce ethanol. The highest ethanol content was produced by *Candida* sp. 1 (56%) while by *Candida* sp. 3 (70%). While the optimum time for the ethanol fermentation process *Candida* sp. 1 and *Candida* sp. 3 is 24 hours.

Keywords: Ethanol; Fermentation; Soil Drive Nutrient System; Sugar; Wild Yeast.

INTRODUCTION

Yeast is one of microorganisms belong to unicellular fungi. Vegetative reproduction in yeast primarily by budding. As single cells, yeast grow faster than mold. Based on the properties of yeast metabolism, yeast can be divided into two groups: fermentative and oxidative yeast. Fermentative types can perform alcoholic fermentation, which breaks down sugar (glucose) into alcohol, lactic acid and gas for example, in bakery products and bioethanol. While oxidative (respiration) can produce carbon dioxide and water (Fardiaz, 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). The ability of the isolate to ferment alcohol has not been studied further.

The purpose of this research is to observe the potential of soil wild yeast that isolated from Jember, East Java in ethanol fermentation and determine the concentration of sugar as well as the appropriate time for the fermentation of ethanol for each isolate.

MATERIAL AND METHODS

Time and Location of the Research.

This research has been took place from January 2015 to January 2016 in Botanical laboratories, Microbiology and Biotechnology laboratories of Biology Department of Institut Teknologi Sepuluh Nopember Surabaya, and Faculty of Agriculture, University of Brawijaya.

Materials.

The equipments used in this research consisted of test-tube, petri dishes, erlenmeyer flasks, hot plate, pipette, measuring cup, beaker glass, autoclave, analitical balance, magnetic strirer, Laminar Air Flow (LAF), spectrofometer GENESYS 108 UV Vis, kuvet, microscope (Olympus Binocular CX22), object glass, rotary shaker, Durham tube, rotary evaporator (EYELA N-1110S-WD), vortex and picnometer.

The materials used were isolates of Candida sp. 1, Candida sp. 2, Candida sp. 3, Debaryomyces sp. and Kloeckera sp. collections of Faculty of Agriculture's of University of Brawijaya Malang; $(NH_4)_2SO_4$, $MgSO_4$, $7H_2O$, KH_2PO_4, 70% alcohol, aquadest, chloramphenicol, yeast malt broth (YMB) media, yeast malt extract agar media, bromothymol blue, pepton water, glucose and lactophenol cotton-blue.

Methods.

Subculture of Yeast Isolate.

The yeast isolates used in this research came from the collection of Agroeco-technology, Faculty of Agriculture, University of Brawijaya Malang. The yeast isolates were subcultured in YMEA medium, then about 5 mL of the sterilized media was poured into the test-tube. The tube was then fixed in a slanted position and left to cool until the medium condensed, then stored under normal temperature. The yeast isolates then inoculated using ose and incubated for 4 days. Successful subculture is indicated by the growth of yeast on YMEA medium.

Observation of Yeast Characteristics.

Microscopic characteristics were determined based on the appearance of the colonies growth on YMEA, consisting of: colony colors, margin shape, elevation, surface, and texture. Microscopical observation was done through microscope and lacto-phenol coloring; macroscopical observation was done firstly by preparing a smear of the breeding upon the object glass previously colored by lacto-phenol, covered, and mixed with a few drops of the immersion oil; whereupon the characteristics of the cell were observed at 400x and 1,000x magnification. For the magnification rate of 400x, the drops of immersion oil were not necessarily required.

The Test on the Potential of Glucose Fermentation.

One ose of 24-48 hours yeast isolates sample inoculated into the testing media containing 1% of glucose on the test-tube. The media is pre-equipped with bromothymol blue as fermentation indicators and then put into a sterilized Durham tube. The tube then homogenized then chloramphenicol was added to serve as antibacterial and peptone water as nitrogen source. Thereafter the media was incubated for 72 hours at 25-28°C. A positive result is indicated with the changing of color (from blue to yellow) of the media and the appearance of gas in the tube. The media's color changing was due to the acidic formation and gas production.

Starter Production.

The starter was created by mixing 3 gram of yeast extract, 3 gram of malt extract, 5 grams pepton, 0.5 grams of chloramphenicol, 1 gram of $(NH_4)_2SO_4$, 0.4 grams $MgSO_4.7H_2O$, and 2 grams KH_2PO_4, all mixed with 1 liter of aquadest. The mixture was heated to boiling point until perfectly mixed. Afterwards the medium was sterilized with autoclave at 121°C, 1.5 atm for 15 minutes, then cooled until it reached room temperature. Thereafter the medium was poured into production bottles, each containing 72 mL, and mixed with 10% (w/v) or 8 mL of physiological water containing the isolate with the OD rate of 0.5. Then the bottles were incubated for 48 hours in rotary shaker before finally subjected to fermentation tests.

The Test of Fermentation on Glucose Substrate

The fermentation medium being used is glucose medium, each on a concentration of 10%, 20% and 30%. Then a mixture of 3 grams yeast extract, 3 grams malt extract, 5 grams pepton, 1 gram $(NH_4)_2SO_4$, 0.4 gram $MgSO_4.7H_2O$, and 2 gram KH_2PO_4 and 1 liter of aquadest was added. Afterwards the medium was sterilized with autoclave at 121°C, 1.5 atm for 15 minutes, then cooled until it reached normal temperature. Thereafter 200 mL of the medium was poured into Erlenmeyer and the fermentation process initiated. This test was performed by inoculating 10% (w/v) yeast isolate, that had inoculated for 48 hours, into the prepared medium. The fermentation itself was performed within 96 hours in different bottles and its ethanol value measured every 24 hours. During the entire fermentation process, the sample was incubated in the rotary shaker.

Measurement of the Residual Reduction Glucose.

The analysis of glucose residue was done use DNS (Dinitrosalicylic acid) method. 0.2 mL of the sample was taken, then put into a new sterilized test-tube then 1.8 mL of aquadest and 2 mL DNS reagent were added. The test-tube was heated for five minutes to stimulate reactions between glucose and the DNS. When the test-tube was finally restored to normal temperature, the sample's absorbance rate was measured at the wave-length of 540 nm by spectrophotometer UV-Vis. The absorbance value was then implemented to the formula of Glucose Standard Curve.

Ethanol Value Measurement.

The ethanol value in this research 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.

Data Analysis.

This research was executed in a descriptive-quantitative manner. The data obtained were the quantities of ethanol concentration value by picnometer and the value of residual glucose by DNS (Dinitrosalicylic acid) method.

RESULTS AND DISCUSSION

Characteristic of Yeast Isolate.

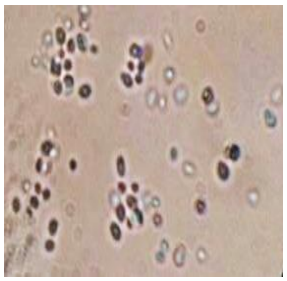
Observations made by looking at the characteristics of the macroscopic colonies grown on YMEA medium. Observation includes colony shape, color of the colony, the colony surface, elevation colonies, the margin of the colony. The test results of macroscopic characters shown in Table 1.

Table 1. Macroscopic Charateristic of *Candida* sp. 1 and *Candida* sp. 3.

Candida **Sp. 3**	***Candida*** **Sp. 1**	**Isolate**
Circular	Circular	Colony Shape
Raised	Convex	Elevation
Undulate	Entire	Margin
White	Cream	Color
Smooth	Smooth	Surface

The next phase was microscopical observation using lactophenol cotton-blue staining and observed through a microscope with a magnification rate of 400x. The staining was the prescribed method to observe microorganisms from Fungi class. Microscopic observation consisted of cell's morphology, budding and whether pseudohypae and/or hyphae was detected (Harley, J. P. and M. Prescott, 2002). The results of microscopic observation of the isolates of *Candida* sp. 1 shows the oval-shaped cells, single cell, and has a multilateral budding (bud is at the tip cell). While in *Candida* sp. 3 shows an oval-shaped cell colony, a single cell and has multilateral budding. It was shown on Figure 1.

a



b

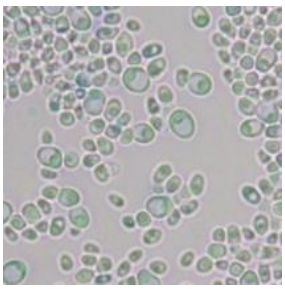


Figure 1. Microscopic Observation of *Candida* sp. 1.

The Test on the Glucose Fermentation Potential.

Glucose fermentation test results showed positive results in *Candida* sp. 1 and *Candida* sp. 3 isolates, which is characterized by a change in color of the medium from green to yellow and also the presence of gas bubbles in the Durham tube. Medium color changed from green to yellow due to the formation of acid and gas production (Wickerham, 1951).

Growth Phase.

It could be inferred from the graphs (Figure 2 and Figure 3) that *Candida* sp. 1 and *Candida* sp. 3 isolates underwent three growth phases. First is the lag phase as the beginning of the yeast's exponential growth. The yeast's cells began to adapt and adjust itself for later self-multiplication. This phase

began from Day 0 to Day 1. The OD of *Candida* sp. 1 on Day 0 was 0.015 and on Day 1, the OD was 1.455. For *Candida* sp. 3, the OD of Day 0 was 0.037 and likewise increased in Day 1 to 1.139. Several factors that contributed to the duration of lag phase are the type and lifespan of the yeast's cells, the number of inoculum and the condition of the growth media. If the yeast's cell grew in a nutrient-lacking media, its duration of the lag phase would be slightly longer. Another parameter that contributed to the length of the phase was the number of inoculum: if a few cells were to be grown in a high-volume media, its lag phase would be longer (Mahreni dan S. Suhenry, 2011).

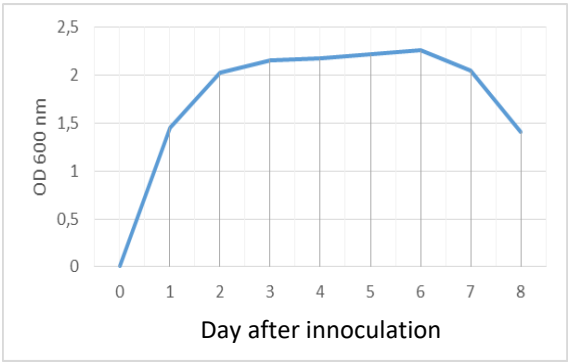


Figure 2. Growth Phase of *Candida* sp. 1

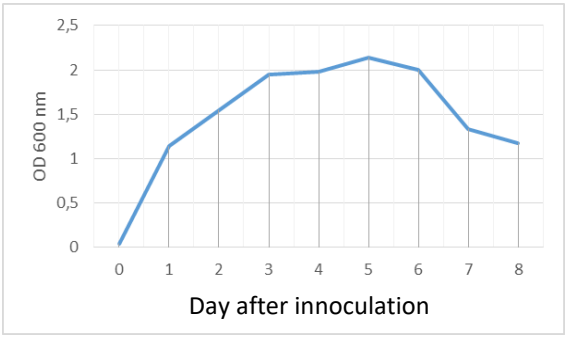


Figure 3. Growth Phase of *Candida* sp. 3

Thereafter both isolats entered log phase. This phase lasted from Day 1-3. During this phase the multiplication of cells occured rapidly coupled with increasing use-up of nutrients, thereby making it the ideal phase for fermentation. Next, from Day 3-6, the yeast cells underwent the stationary phase. The cell population gradually stabilized and there was but small increase of it before the death phase. At this stage the fermentation process slowed down with the entire glucose already used up thus entering the respiration phase. During its stationary phase the yeast's cells would secrete secondary metabolits to defend itself against the oxidatic stress due to accumulations of toxic secondary metabolism products (Lisnawati Y., 2004).

Measurment of Ethanol and Residual Reduction Glucose 24-hour Fermentation.

Table 2. Ethanol and Residual Glucose Values at 24-hours Fermentation.

Isolate	Medium Concentration (%)	Residual Glucose Before (%)	Residual Glucose After (%)	Used Glucose (%)	Ethanol (%)
Candida sp.1	10	4.13	3.70	0.43	0
	15	4.96	3.13	1.56	2
	20	5.88	4.42	1.46	0
Candida sp.3	10	4.13	3.55	0.68	0
	15	4.96	1.98	2.71	70*
	20	5.88	4.08	1.80	0

From table 2, it could be inferred the results of reduction glucose used up for 24 hours. At the 10% concentration medium *Candida* sp. 1 used up 0.78% of glucose and produced 0.8% of ethanol. *Candida* sp. 3 used up 0.62% of glucose and didn't produced ethanol. At the medium concentration of 15% *Candida* sp. 1 used up 0.92% of glucose and produced 56% of ethanol; *Candida* sp. 3 used up 3.11% of glucose and produced 3.4% of ethanol. Then at the concentration medium of 20%, *Candida* sp. 1 used up 0.56% of glucose and didn't produced ethanol and *Candida* sp. 3 used up 0.06% of glucose and didn't produced ethanol.

The concentration of glucose will drop gradually during the fermentation process in relation with the increase of cell and etanol production. This is because cells consumed glucose within the system to increase cell growth and etanol production. When yeasts produce etanol, the amount of glucose consumed will proportionately be higher (Cheng et al., 2009).

Candida sp. 1 can produce ethanol at a concentration of 10% by using sugar as much as 0.78%. 0.78% of the sugar will be used to cell respiration and will be converted into ethanol as a fermentation product. While in *Candida* sp. 3 using sugar as much as 0.62%, is not capable of producing ethanol in medium 10%. Its because both isolates have a different intake of glucose in their metabolism.

At medium concentrations of 20%, both isolates were not able to produce ethanol. The sugar concentration generally made in the manufacture of ethanol is about 14%-20%. If the sugar concentration is too high, it will inhibit yeast activity (Judoamidjojo, 1990). Decreased levels of ethanol in excess glucose concentrations occur as the inhibitory effects of substrate and product. High substrate concentration will reduce the amount of dissolved oxygen at medium (Elevri, P. A. and S. R. Putra, 2006). This is causing isolates of *Candida* sp. 1 and *Candida* sp. 3 is not able to produce ethanol at a medium concentration of 20%.

48 hours Fermentation.

From the table, it could be inferred the results of reduction glucose used up for 48 hours. At medium concentrations of 10%, *Candida* sp. 1 used glucose as much as 0.43% and didn't produced ethanol while at *Candida* sp. 3, used glucose as much as 0.68% and didn't produced ethanol. Then in the medium concentration of 15%, *Candida* sp. 1 used as much as 1.56% glucose and produced 2% ethanol, while *Candida* sp. 3, used glucose as much as 2.71% and produced 70% ethanol. Then, in the medium concentration of 20%, *Candida* sp. 1 used glucose sugar as much as 1.46% and didn't produce ethanol, while the *Candida* sp. 3 used glucose as much as 1.80% and didn't produced ethanol.

Table 3. Ethanol and Residual Glucose Values at 48 hours Fermentation.

Isolate	Medium Concentration (%)	Residual Glucose Before (%)	Residual Glucose After (%)	Used Glucose (%)	Ethanol (%)
Candida sp.1	10	4.13	3.35	0.78	0.8
	15	4.96	4.02	0.92	56*
	20	5.88	5.32	0.56	0
Candida sp.3	10	4.13	3.51	0.62	0
	15	4.96	1.85	3.11	3.4
	20	5.88	5.82	0.06	0

In the 48 hours fermentation, isolates of *Candida* sp. 1 and *Candida* sp. 3 is only able to produce ethanol at 15% concentration of medium. At medium concentrations of 10%, glucose consumption is only slightly 0.43% by *Candida* sp. 1 and 0.68% in *Candida* sp. 3. This is due, in 48 hours, yeast also produces secondary metabolites such as acetic acid. In the first stage, glucose is converted to ethanol and acetic acid. After that, most of the ethanol also oxidised to acetic acid. These acids continue to accumulate after the glucose is exhausted and ethanol oxidation takes place. After depletion of glucose, ethanol is oxidized to acetic acid, acetic acid, and finally consumed and oxidized to CO_2 and water (Van Dijken, J. P. and W. A. Scheffers, 1986).

72 hours Fermentation.

From table 4, it could be inferred the results of reduction glucose used up for 72 hours. At medium concentrations of 10%, isolates of *Candida* sp. 1 used glucose as much as 0.39% and didn't produced ethanol, while the isolates of *Candida* sp. 3 used glucose as much as 0.63% and didn't produce ethanol. Then in the medium concentration of 15%, *Candida* sp. 1 used glucose as much as 1.02% and didn't produced ethanol, while *Candida* sp. 3, used glucose as much as 1.23% and didn't produced ethanol. Then in the medium concentration of 20%, *Candida* sp. 1 used glucose as much as 1.33% and didn't produced ethanol, while *Candida* sp. 3, used glucose as much as 1.33% and didn't produced ethanol.

In the 72 hours fermentation, two isolates didn't produce ethanol at all. Because oxygen levels in the medium already be drained so that isolates can not perform respiration and fermentation. Yeast fermentative have different responses to environmental conditions with respect to alcoholic fermentation. Many species of yeast capable of fermenting alcohol only under the condition of no oxygen but in small amounts and not anaerobic. Oxygen is needed for glucose fermentation in yeast cells, as in the absence of oxygen they will not grow on glucose (although oxygen is not required for the conversion of glucose into ethanol) Van Dijken, J. P, A.W. Ruud and T. P. Jack, 1993).

Table 4. Ethanol and Residual Glucose Values at 72-hour Fermentation

Isolate	Medium Concentration (%)	Residual Glucose Before (%)	Residual Glucose After (%)	Used Glucose (%)	Ethanol (%)
Candida sp.1	10	4.13	3.74	0.39	0
	15	4.96	3.94	1.02	0
	20	5.88	4.55	1.33	0
Candida sp.3	10	4.13	3.50	0.63	0
	15	4.96	3.73	1.23	0
	20	5.88	4.51	1.37	0

96 hours Fermentation.

Table 5. Ethanol and Residual Glucose Values at 96-hour Fermentation.

Isolate	Medium Concentration (%)	Residual Glucose Before (%)	Residual Glucose After (%)	Used Glucose (%)	Ethanol (%)
Candida sp.1	10	4.13	3.61	0.52	0
	15	4.96	4.37	0.59	0
	20	5.88	4.95	0.93	0
Candida sp.3	10	4.13	3.47	0.66	0
	15	4.96	3.98	0.98	0
	20	5.88	4.13	1.75	0

From Table 5, it explained that at medium concentrations of 10%, *Candida* sp. 1 used up glucose as much as 0.52% and didn't produced ethanol, while the *Candida* sp. 3, used glucose as much as 0.66% and didn't produced ethanol. Then in the medium concentration of 15%, *Candida* sp. 1 used glucose as much as 0.59% and didn't produced ethanol, while the isolates of *Candida* sp. 3 used up glucose as much as 0.98% and didn't produced ethanol. Then in the medium concentration of 20%, *Candida* sp. 1 used glucose as much as 0.93% and didn't

produced ethanol, while *Candida* sp. 3 used up glucose as much as 1.75% and didn't produced ethanol.

At 96 hours fermentation, two isolates did not produced ethanol at all. The same thing happen on the 72 hours of fermentation occurs. Isolates of *Candida* sp. 1 and *Candida* sp. 3 didn't produced ethanol by fermentation treatment 96 hours in each medium concentration. Oxygen levels in the medium is drained so isolates can not perform respiration and fermentation. Oxygen is necessary for the growth of yeast as a building block for the biosynthesis of unsaturated fats and lipids needed in mitochondrial and plasma membrane. The consequences of the lack of oxygen is limiting the growth of yeast cells, reducing cell viability and fermentation is slow and incomplete (Arifa, A. Madiha, and F. Tasnim, 2010).

CONCLUSION

Both *Candida* sp. 1 and *Candida* sp. 3 has a good potential to produce etanol. The proper glucose medium concentration for the fermentation of both species in this research is 15%. The optimum time needed for fermenting the yeasts of both species was 24 hours. The highest ethanol content produced by *Candida* sp. 1 is 56% while in *Candida* sp. 3 is 70%.

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