Isolation And Characterization Of Fermenting Yeast From Traditional Ethanol Production

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Abstract. Ethanol fermentation still plays a significant role in the industry, as it has a huge economical value. Saccharomyces cerevisiae is the most often culture being used for this fermentation because its superiority in producing ethanol. However, this yeast also showed its limitation because of the toxic effects of ethanol to the cells. This research was done to isolate new fermenting yeasts from traditional fermentation process in Solo, Central Java, with the hope to obtained more potential strains. A series of isolation and purification steps were done and two potential yeasts isolates obtained. They are identified as Schizosaccharomyces pombe and Zygosaccharomyces parabailii, respectively. At least one isolates showed comparable ethanol fermentation capacity compared to S. cerevisiae and deserve further research.

1. Introduction
Ethanol fermentation could be considered one of the oldest cultivation method in man’s history. Researches, reports, as well as reviews have been produced related with this fermentation. Saccharomyces cerevisiae is the most studied fermenting yeast which perform anaerobic facultative process [10], it is also the most common strain used for ethanol fermentation as it high osmotic tolerance, as well as higher sugar conversion into ethanol [4].

Despite this rapid ethanol conversion, it is reported elsewhere that ethanol itself has deleterious effect on the producing yeast [5], as the fermentation is influenced by factors such as type of substrate, final ethanol concentration, oxygen contents, and culture being used [3]. [7] reported the denaturation of glycolytic enzymes due to the high level of ethanol resulting in the reduction of biomass growth and glycolytic activity. It is already observed the organel damaged that cause in the reduction up to growth termination due to inhibition effect of ethanol [1]; [3]; [7]. The higher ethanol content in the fermentation medium, the more severe damage observed to the cells; 5-7% ethanol content may result in cell growth reduction, while 10% ethanol may cause protein denaturation which in turn will resulting in cell mortality [8].

Researches have been done to overcome the problem, such as the use of specific fermenter tank to continuously removed the ethanol content from the fermentation medium [11], which in turn could enhanced ethanol yield up to 31.3%. Another approach have been done by [9] who basically destroy the autophagy system, especially the mitophagy in S. cerevisiae. Mitophagy is known as selective form of autophagy that degrade mitochondriae of S. cerevisiae, which resulting in the improvement of ethanol yield up to 2.12%.
As the ethanol effects on the fermenting yeasts are still need to be overcome, however, another approach could be done to improve the ethanol fermentation efficiency. It is reported that the laboratory strains behave differently as compared to its wild-type. As reported by [6] that working culture which are used in the laboratory may have not represent the optimum potency as compared to the wild-type S. cerevisiae.

Based on the above mentioned background, it is the aim of this research to isolate yeast strain(s) from traditional ethanol fermentation, and characterize its fermentation kinetics in attempt to improve ethanol yield from sugar fermentation.

2. Research Methods

2.1. Isolation of yeasts from traditional ethanol fermentation
Isolation was done by collecting “laru” samples of “Ciup” production from Bekonang, Solo, Central Java. A series of dilution was done and continued with plating on PGY with or without antibiotics addition, i.e. chloramphenicol and tetracycline with the concentration of 75 and 100 ppm, respectively.

2.2. Yeast regeneration and maintenance
Cells of S. cerevisiae D-01, considered as working culture, and isolated yeasts, as wild-type, are maintain and grown on PGY medium.

2.3. Characterization
The obtained isolates were characterized by observing their macroscopic and microscopic morphology, for examples the colonies colour, cell size, cell shape and budding type.

2.4. Antagonistic assay
The assay was basically done by streaking the obtained isolates and observed for the inhibition zone against the test organisms.

2.5. Fermentation
Fermentation were done by inoculating 10% starter to the fermentation media of 10-15% glucose, placed on a rotary shaker for 4 days at room temperature. Cell biomass were basically measured as cell number (CFU/ml) by plating 1 ml samples continued by serial dilution, and plating on PGY medium. Sugar content was measured by employing DNSA method by collecting 0.3 ml samples, added with 0.3 ml DNSA reagent, added with 2.4 ml water and heated for 10 min before taking OD measurement at 540 nm. Ethanol concentration was determined by GC analysis by sending the samples to UII, Yogyakarta. 30 ml samples were distilled until it reached 10 ml, placed in an eppendorf and tightly sealed before being sent for measurement.

2.6. Molecular identification of obtained isolates
Obtained isolates were further characterized by conducting DNA barcoding, i.e. by sending the samples to be sequenced. The isolates were send to Macrogen, Korea and sequenced for 16S rRNA. After obtaining the sequences, data were analysed by conducting some bioinformatics analysis using MEGA software and comparing the sequences with the online database, i.e. GenBank.

3. Results and Discussion
Isolation of yeast(s) from laru obtained from Bekonang, Solo, Central Java resulting in several different yeast colonies based on their morphological appearance, and the addition of antibiotics effectively prevent the occurrence of bacteria. Based on this observation, 17 different yeasts were obtained. Figure 1 shows typical colonies obtained on media either with or without antibiotics addition. It is worth to mention here that the strains in Fig.1 are the most often and dominant colonies obtained in isolation steps as described in Materials and Methods.
After following several purification steps, a single colony could be recovered and further examination be performed. The resulting colonies are presented in Figure 2, shows that the small colony are more whitish in colour compared to the bigger colony.

![Figure 1](image1.png)

**Figure 1.** Yeasts (a) big and (b) small colonies, obtained from isolation steps grown on PGY media

Subsequently, a microscopic examination was done to further characterize both isolates obtained. The resulting observation is presented in Figure 3 and Table 1, showing that the two colonies are differ in cell shape, colour and its budding type.

![Figure 2](image2.png)

**Figure 2.** Single colonies from both (a) small and (b) big colony grown on PGY medium

As can be seen in Fig.3 and Table 1, the small colony has an ellipse shape, lighter in colour and with bipolar budding type, as compared to the bigger colony which has a smaller cell size, round-ellipse cell shape and darker, and unipolar budding type. Based on the observed data, it is most probably that the two isolates may belong to the different species/strain, regardless the difficulty to separate these two isolates during the isolation and purification steps. The next step will be molecularly identify the obtained isolates.

![Figure 3](image3.png)

**Figure 3.** Microscopic properties of (a) bipolar budding of the small colony and (b) monopolar budding for the big colony (magnification: 400x)
Table 1. Selected macroscopic and microscopic properties of obtained isolates

| Colony | Shape       | Cell colour       | Budding     |
|--------|-------------|-------------------|-------------|
| Small  | Ellipse     | Transparent       | Bipolar     |
| Big    | Round-ellipse | Dark transparent | Monopolar   |

In parallel to the molecular identification, a series of antagonistic assays were done to further characterize whether these isolates have the ability to inhibit the growth of other organisms. The results could be seen in Figure 4, showing that there are no antagonistic activity from both of the isolates.

![Figure 4](image)

**Figure 4.** A typical antagonistic assay of the isolates obtained showing no antagonistic activity; (a) both colonies are grown on plate spread with laru; (b) small colony grown on lawn of big colony, and (c) big colony grown on lawn of small colony

Molecular identification of both isolates based on their 16S rRNA resulting in the identification of two different species, as presented in Table 2, namely *Schizosaccharomyces pombe* and *Zygosaccharomyces parabailii*, respectively. It was reported that *S. pombe* was also recognized from laru of Bekonang, Solo, and considered one of the most active fermenting yeast resulting in high ethanol content compared to other microorganisms found, either yeasts, fungi and bacteria [2].

Table 2. Molecular identification of obtained isolates

| No. | Colony | Species                       | Similarities | Acc. No.   |
|-----|--------|-------------------------------|--------------|------------|
| 1.  | Small  | *Schizosaccharomyces pombe* strain BY-10 | 95%          | KJ562356.1 |
| 2.  | Big    | *Zygosaccharomyces parabailii* ATCC 56075 | 95%          | JX458094.1 |

To test the finding, we performed fermentation with 12.5% glucose by using *S. cerevisiae* D-01 which is considered as working culture, as it is normally being used in ethanol fermentation in the laboratory, and compared it with the two isolates obtained in this research. The results are presented in the Figure 5 and Figure 6 below.
Figure 5. Fermentation performance of *S. cerevisiae* D-01 for 4 days on PGY medium containing 12.5% glucose

Figure 6. Fermentation performance of isolates obtained on 12.5% glucose

The pictures on Fig.5 and Fig.6 clearly shows that at least the small colony obtained from laru and identified as *S. pombe* give a comparable amount of ethanol yield compared to *S. cerevisiae* D-01. Further researches are still going on to better characterize the isolates obtained, as well as to test their ability to produce ethanol compared to laboratory strains. More data are needed to better analyze the fermentation kinetics of the isolates being used.

4. Conclusion

Based on our findings, two yeasts species identified as *S. pombe* and *Z. parabailii*, respectively, are recovered from laru. The isolates showed comparable fermentation capability compared to working culture, based on the ethanol yield.

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