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SCREENING OF FLOCCULANT *SACCHAROMYCES CEREVISIAE* (NCYC 1195) FOR HIGH TOLERANCE OF ETHANOL CONCENTRATION

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***Abstract*---Yeast are exposed to various stresses during ethanol fermentation. Among the stresses, ethanol is considered to be major stress responsible for decrease ethanol production. At concentration in excess 8% (v/v) ethanol cause the phospholipid of the lipid bilayer of cell membrane and organelles become more permeable to small molecules and ions, the perturbation of cell homeostatis impacts on several cellular metabolic pathway. This study was aimed to the increasing in ethanol tolerance of *Flocculant Saccharomyces cerevisiae* (NCYC 1195) by random gamma mutagenesis. The yeast was irradiated with gamma ray and screened for their ability to grow at high ethanol concentration. The yeast was placed in a sterile distillated water, and irradiated at different dose (0.6, 0.8, 1, 1.2, and 1,4 KGy). Among 6 ethanol tolerant mutants, Y7 and Y8 showed highest ethanol tolerance up to 5% (v/v) ethanol concentration, while the wild type had ethanol tolerance only 2.5% (v/v) ethanol concentration. The Y8 produced 15.9% more viable cells than wild type with lowest glucose consumption when its fermentation ability tested in YPG broth medium containing 10% glucose. Thus Y7 and Y8 would be good candidate to be a high ethanol producer.**

Keyword*---Ethanol tolerance, gamma mutagenesis, flocculant *Saccharomyces cerevisiae

I. Introduction

Recently, major thrust has laid upon production of biofuels instead of fossil fuels due to constantly decreasing amount of fossil fuel reserves and the dilemma that when non-renewable energy will be depleted. Bioethanol has been regarded as a favorable alternative energy source, which is both renewable and environmental friendly [1]. The fermentation process of ethanol production is commonly done by yeast. Increasing the availability of this alternative energy source requires ethanologenic yeasts that can produce ethanol more efficiently [2].

Saccharomyces cerevisiae has been broadly used for fuel ethanol production due to its ability to produce high concentration of ethanol from simple sugars. The self-flocculating yeast strains produce higher cell concentration under proper condition in the bioreactor and provide efficient separation process of yeast cells from fermenting mash at the end of fermentation [3]. Ethanol counts as a toxin for yeast cells and tolerance to it is closely related to ethanol productivity which is a major factor in industrial ethanol production [4]. At concentrations in excess of 8% (v/v) ethanol cause the phospholipid of the lipid bilayer of cell membranes and organelles, such as the inner membrane of mitochondria, to become hyperpolarized thereby increasing membrane fluidity and consequentially decreasing membrane integrity [5]. When the cell membrane becomes more permeable to small molecules and ions, the perturbation of cell homeostasis impacts on several cellular metabolic pathways [6]. Improving and increasing understanding of the impact of ethanol toxicity on yeast cells will assist enhancing yeast ethanol tolerance and higher ethanol production [7]. Consequently, several studies to date have focused interest on ethanol tolerance of ethanol-producing yeasts based on the presumption that ethanol-tolerant yeast strains would have enhanced ethanol productivities and yields [8].

The process of increasing ethanol tolerance of yeast can be done by some mutation technique. Earlier studies showed that mutations induced by ^{60}Co Gamma ray in *Saccharomyces cerevisiae* can improved tolerance ability of yeast to high concentration of ethanol [9]. Induction of mutation revealed that the survival percentages were decreased by increasing the dose of gamma rays, while the mutant percentages were increased by increasing the radiation intensities, that is, doses [10]. High ethanol tolerant strains are able to extend the process of fermentation for longer time and produce distinct products in the presence of ethanol.

The main objective of this study was to increase the tolerance ability of flocculant *Saccharomyces cerevisiae* (NCYC 1195) to high concentration of ethanol. Fermentation characteristics of the ethanol-tolerant mutants in YPG broth medium were examined in comparison to the wild-type.

II. Materials and Methods

A. Strain

The flocculant yeast strain (NCYC-1195) was obtained from the National Collection of Yeast Cultures, Institute of Food Research, Norwich Research Park, Norwich, United Kingdom.

B. Media

The flocculant yeast strain (NCYC-1195) maintained in yeast extract peptone glucose (YPG) broth medium, containing yeast extract (1% (w/v)), peptone (2% (w/v)), and glucose (2% (w/v)), was obtained from the available stock culture of food microbiology laboratory, Brawijaya University, Malang, Indonesia. Yeast storage stock solution were prepared by adding 50% (v/v) glycerol to growth YPG broth medium of yeast after 24 hours and were store at -30^{O}C. When required, the yeast was activated by transferring to YPG broth medium (pH 5). The culture was incubated at 30^{O}C on a waterbath shaking at 120 rpm.

C. Radiation

One ml of yeast cell suspension (approximately 1 x 10^8 cell/ml) was radiated with ^{60}Co as a source of Gamma ray at different dose (0.6, 0.8, 1, 1.2, and 1,4 KGy/h). The aim was to identiy highest lethality and was obtained by plating and incubating of radiated yeast cell suspension at 30^{O}C. The growing colonies were isolated after 24 hours.

D. Screening of Ethanol-Tolerance Mutants

About 0.1 ml of the cell suspension (1 × 10^8 cell/ml) was added to 5 ml YPD liquid and cultivated at 30°C overnight. The cells were exposed to gradual additions of ethanol (96.0%) to cultures. Concentrations were raised from 0% to 10% (v/v) within 4 days (additions were 2.5% ethanol for each day). Cultures were plated on YPG agar before ethanol addition each day. The growing colonies were isolated after 48 hours incubation at 30^{O}C.

E. Testing Growth Profile

One loop of yeast cell was inoculated to 5 ml activation culture and incubated for 20 hours at 30^{O}C with 120 rpm shaking. About 0.1 ml of activation culture was transferred to 5 ml seed culture and incubated for 20 hours at 30^{O}C with 120 rpm shaking. Seed culture was centrifuged at 7000 rpm for 10 minutes and pellet was taken. The pellet was resuspension with sterile YPG broth and transferred to 95 ml YPG broth fermentation medium containing 10% glucose. Growth profile testing was performed at 30^{O}C for 72 hours with 120 rpm shaking. Fermentation samples was taken every 12 hours for determining viable cell count, reducing sugar, and potential of hydrogen (pH) in the culture.

F. pH and Reducing Sugar Analysis

3 ml of samples were centrifuged at 7000 rpm for 10 minutes. The supernatant was taken and used to determined the pH of fermentation medium. Then, supernatant was neutralize by addition of 3 M NaOH solution. One ml of supernatant was diluted until 250 fold. Two ml of DNS was added to two ml of diluted supernatant. The mixture was vortexed, and then heated at 100^{O}C for 10 minutes. The heated mixture was cooled with cold water. Then, the optical density of mixture was measured with spectrophotometer.

G. Viable Cell Count Analysis

1 ml of samples were centrifuged at 7000 rpm for 10 minutes. The pellet was taken and resuspension by addition of deflocculation buffer. The optical density of suspension was measured with spectrophotometer.

III. Results and Discussion

A. Effect of Gamma Ray Treatments on Yeast

Ethanol tolerance of yeast cell is closely related to ethanol productivity. In the present study, an attempt was made to carry out the ethanol tolerance improvement of flocculant *Saccharomyces cerevisiae* for increased ethanol production. Ionizing radiation with gamma ray is a physical method that used widely for creating mutagenesis [10]. Selected yeast suspension was irradiated by different doses of gamma (γ) ray. The irradiation result show that the gamma radiation excess 0.6 KGy caused high yeast cell lethality. At 1 KGy, there was no yeast cell growth found (Table 1.). The result confirmed the radiosensitivity of *Saccharomyces cerevisiae* W303-1A and BY4741 Strains work [11].

Table 1. Number of survival colonies by different doses of gamma ray

Dose (KGy)	Survival Colonies (CFU/ml)
0	44 x 10^6
0.6	26
0.8	2
1	0
1.2	0
1.4	0

Data (Table 1.) show that there was 26 survival colonies obtained after gamma ray treatments at 0.6 KGy dose, 2 survival colonies at 0.8 KGy dose and there was no survival colonies obtained excess 1 KGy dose.

B. Screening of Ethanol-Tolerance Mutants

Table 2. Number of screened colonies and ethanol-tolerance mutant

Dose (KGy)	Number of Screened Colonies	Mutants
0.6	26	4
0.8	2	2

The survival colonies from gamma ray treatment were screened for its tolerance ability in the presence of ethanol. There was only 4 mutants (Y6a, Y6b, Y6c and Y6d) from 0.6 KGy dose that could survive in the presence of 2.5 % (v/v) ethanol concentration and 2 mutants (Y7 and Y8) from 0.8 KGy dose that could survive in the presence of 5 % (v/v) ethanol concentration, while the wild type could survive in the presence of 2.5 % (v/v) ethanol concentration. Mutant percentages were increased by increasing the radiation dose, while the survival percentages were decreased [10]. These 6 mutant were isolated and preserved for testing its fermentation ability. Among the mutants and wild type, mutant Y7 and Y8 would be good candidate to be a high ethanol producer than the other because its tolerance ability.

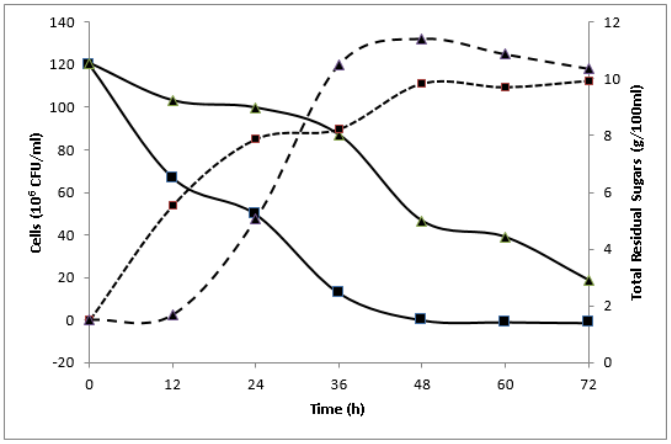

Figure 1. Changes in measured parameters of viable cell count (dashed lines) and residual sugars (solid lines) during YPG broth medium containing 10 % glucose fermentation of Y8 (filled triangle) and the wild-type (filled square). All experiments were performed at 30°C with 120 rpm.

C. Growth Profile

The cell viability, growth and sugar consumption in YPG broth medium containing 10 % glucose of the ethanol-tolerant mutant, Y8, along with the wild-type were investigated further. The result (Figure 1.) show that mutant Y8 displayed higher numbers of viable cells than the wild type during YPG broth medium containing 10 % glucose fermentation. The highest value of a viable cell count of Y8 was found at 48 h of fermentation, which was 15.9% higher than the wild-type. While the ability of mutant Y8 to utilize sugar in YPG broth medium were lower than those of the wild-type. The mutant Y8 displayed a slow growth rate and slow sugar consumption in the early fermentation. After 24 h of fermentation, the mutant Y8 have increased its growth rate significantly. While the wild type showed more stable growth rate from early fermentation until end of fermentation. Growth profile of mutant Y8 showed that this mutant Y8 would be good candidate to be high ethanol producer. Yeast cells with high cell viability and rapid growth are the important factors to increase ethanol production rate.

IV. Conclusion

The present study shows that flocculant *Saccharomyces cerevisiae* (NCYC 1195) have highest cell lethality by gamma irradiation treatments at 0.6-0.8 KGy. Mutagenesis by gamma irradiation treatments made it possible to find two mutants, Y7 and Y8, with high ethanol tolerance (5 % ethanol concentration (v/v)) than the wild type and four mutants (Y6a, Y6b, Y6c and Y6d) that have similar tolerance ability in the presence of 2.5 % (v/v) ethanol concentration. Mutant Y8 display higher numbers of viable cell than the wild type, but lower sugar consumption during (10% glucose) YPG broth medium fermentation.

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FUTURE RESEARCH

In the next study, we will determine the optimum fermentation condition for ethanol production and investigated the stability of mutants.

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