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The effects of stress-induced mutation using EMS on ethanol tolerance in *Saccharomyces cerevisiae*

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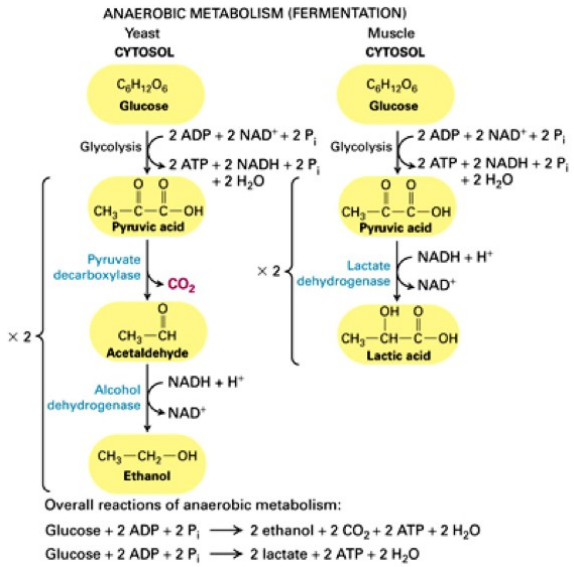
ABSTRACT

Ethanol is a multifunctional compound that has many uses and can be made naturally by sugar-fermenting yeast such as *Saccharomyces cerevisiae*. However, ethanol is toxic to yeast, so the process is not as efficient as it could be. This research aims to identify an efficient mechanism to increase the ethanol tolerance in *Saccharomyces cerevisiae* using various techniques such as ethanol as a stressor, artificial selection, and ethyl methanesulfonate (EMS) as a chemical mutagen. *Saccharomyces cerevisiae* cells were repeatedly exposed to increasing levels of ethanol from 9%-27% and EMS. The parental strain was defined to have an ethanol tolerance of 13%; the artificial selection strain (only exposed to ethanol as a stressor and artificially selected after each round) was defined to have an ethanol tolerance of 16%; the EMS-exposed strain (also exposed to ethanol as a stressor and artificial selection) was defined to have an ethanol tolerance limit at 27%. To test differences between strains, the parental strain, selection strain, and the EMS-exposed strain were separately plated on ten 27% ethanol plates and ten 0% ethanol plates and growth was checked after 24 hours. The EMS-exposed strain was the only strain that grew at 27% ethanol; all strains grew at 0% ethanol. These results show that EMS, artificial selection, and ethanol as a stressor might be effective in producing strains of *Saccharomyces cerevisiae* that are able to produce greater amounts of ethanol before toxicity sets in.

Keywords: *Saccharomyces cerevisiae,* Ethyl methanesulfonate, artificial selection, ethanol tolerance, chemical mutagenesis, molecular genetics, microbiology

INTRODUCTION

Saccharomyces cerevisiae is a yeast species that is capable of fermenting simple sugars such as glucose into ethanol. The pathway used to produce ethanol in yeast follows the glycolysis pathway in anaerobic conditions and transforms glucose into pyruvate. From pyruvate, humans can produce lactic acid in anaerobic muscle tissues, but yeast turn pyruvate into acetaldehyde and then into ethanol following the pathway below:



http://fhs-bio-wiki.pbworks.com/w/page/12145772/Fermentation

Although *Saccharomyces cerevisiae* can produce ethanol from many different sugars, the ethanol produced is lethal to the yeast cells in higher concentrations (Ma & Liu, 2010). Accumulation of ethanol can wreak havoc on the cells by inhibiting glucose uptake, deteriorating cell membrane functions, causing protein denaturation and conformation issues, and a host of other detrimental effects (Stanley et. al., 2009).

Although ethanol can be detrimental to yeast in higher concentrations, there are many advantageous uses of bioethanol, and perhaps, the most well-known use is the fermentation process in fruit and grains to produce alcoholic beverages. Another, more recent, beneficial use is the production of bioethanol from cellulosic starches for use as a biofuel that is considered clean and renewable (Ma & Liu, 2010). One breakthrough for this area of science was the use of ethanol to make E85 (85% ethanol) as a fuel to power automobiles. It is important to the future of our planet to continue to seek out renewable energy sources such as biofuels that don't attribute to greenhouse gases, don't focus on using precious natural resources, and don't contribute to global warming. Other uses for ethanol include antiseptics, antidotes to methanol and ethylene glycol poisoning, solvents for water-insoluble agents, and household heating.

Ethanol tolerance in *S. cerevisiae* can vary depending on the strain, but the average upper-limit tolerance is around 15-16%. Researchers have recently been interested in determining the

mechanisms behind ethanol tolerance in yeast cells so that they can be modified to produce higher concentrations of the substance and still survive. One way of doing this is introducing positive mutations in genes responsible for ethanol tolerance, however, there isn't enough evidence to see exactly which genes are affecting ethanol tolerance the most. It is likely due to an effort of multiple genes and regulators (Ding et. al., 2009). Over 400 genes have been linked to the process (Ma & Liu, 2010 (quantitative)). A few of the main family of genes identified in ethanol tolerance in yeast are the heat shock protein genes and cell membrane protein genes (Ma & Liu, 2010 (quantitative)). Instead of performing direct mutagenesis within a specific gene using genome editing techniques that can be costly, EMS (ethyl methanesulfonate) can be used as a mutagenic agent that is capable of producing genome-wide SNP's (single nucleotide polymorphisms) that is relatively inexpensive (Shirasawa et. al., 2016). EMS is capable of producing transition, insertion and deletion mutations and works by acting as an ethylating agent that attacks nitrogen positions in the bases in DNA, and it is also capable of alkylation of oxygens in guanine bases (Sega, 1984). By subjecting yeast cells to a mutagen and increasing levels of ethanol, the cells will become stressed out and will be readier to adapt and turn on stress-response mechanisms to induce higher ethanol tolerance (Hemmati et. al., 2012).

I expect the selective engineering techniques along with the EMS exposure will be an effective mechanism to produce a genetic change at the molecular level in the *S. cerevisiae* treatment strain that enables the cells to have increased ethanol tolerance, and it will be able to produce more ethanol per gram of glucose consumed so the process will become more efficient.

MATERIALS AND METHODS

Strain and Media

The parental strain used was a *Saccharomyces cerevisiae* haploid wild-type strain purchased from Carolina Biological. Fresh stock cultures were grown in Yeast Extract/Peptone/Dextrose (YPD) broth at 30°C for 24 hours. The parental stock was kept at 4°C for later use. YPD agar with varying concentrations of ethanol was used for plating techniques.

Treatment Groups

Three different treatment groups were used to determine which method to increase tolerance would be the most effective. The control group was the parental strain with natural variation. The selection strain was exposed to artificial selection and ethanol as a stressor only. And the EMS-exposed strain was exposed to artificial selection, ethanol as a stressor, and EMS as a chemical mutagen.

EMS Exposure

I added 1 mL of the stock culture and 1 mL 4% EMS to 10 mL of fresh broth. This mixture was vortexed for 10 seconds and placed in a shaker incubator at 30°C with gentle agitation for 40 minutes. 1 mL of 5% Sodium Thiosulfate was added to the mixture and vortexed to stop the effects of EMS. The mixture was then centrifuged and washed again with 5% Sodium Thiosulfate. The cells were pelleted and resuspended in 10 mL of fresh broth.

Plating

I spread 20 microliters of the EMS exposed cells on YPD agar plates with varying concentrations of ethanol. For the first round of plating, I used ethanol in 0%, 9%, 12%, and 15% concentrations. The parental strain was also plated on those concentrations as a control. Plates were placed in a 30°C incubator and growth was checked after 24 hours.

Repeat Rounds

Surviving EMS-exposed cells from the highest ethanol concentration plate were selected and resuspended in 50 mL of fresh broth and grown overnight in a shaker incubator at 30°C. EMS exposure was repeated, and the second-round concentration agar plates were 0%, 13%,16%, and 19%. Growth was checked after 24 hours. Selection of cells, resuspension and growth, and EMS exposure were repeated, and third-round concentration agar plates were 0%, 17%, 20%, and 23%. Growth was checked after 24 hours. Selection of cells, resuspension and growth, and EMS exposure was repeated, and fourth-round concentration agar plates were 0%, 21%, 24%, and 27%.

Parental strain cells from the highest ethanol concentration plate were selected and resuspended in 50mL of fresh broth and grown overnight in a shaker incubator at 30°C. This strain became the new artificial selection strain that was only exposed to increasing ethanol as a stressor, but not the EMS. Percentages for each round of increased ethanol was the same as described above in EMS rounds.

Testing Differences

EMS exposure ceased after four rounds, and an upper limit concentration tolerance for the mutated cells was defined at 27%. Parent cells, artificial selection cells, and EMS-exposed cells were plated separately on ten YPD agar plates each at the defined upper-limit concentration of 27% and ten 0% plates. The cells grew at 30°C for 24 hours and growth was checked.

RESULTS

The upper-limit tolerance of the parental strain was defined at 13%. The upper-limit tolerance of the artificial selection strain exposed only to ethanol as a stressor was 16%. The upper-limit tolerance of the EMS-exposed cells was defined at 27%. The parental strain did not grow on any of the 10 plates at 27%; the selection strain did not grow on any of the 10 plates at 27%; the EMS-exposed strain grew on 10 of the 10 plates at 27% (χ^2=30.00, df=2, p=<.001). All three of the strains grew on all plates at 0% concentration (χ^2=30.00, df=2, p=1.00). These results show that the combining effects of ethanol as a stressor and EMS as a chemical mutagen altered the parental strain at the molecular level to allow for increased ethanol tolerance in the environment. Ethanol as a stressor alone also showed to increase the tolerance slightly.

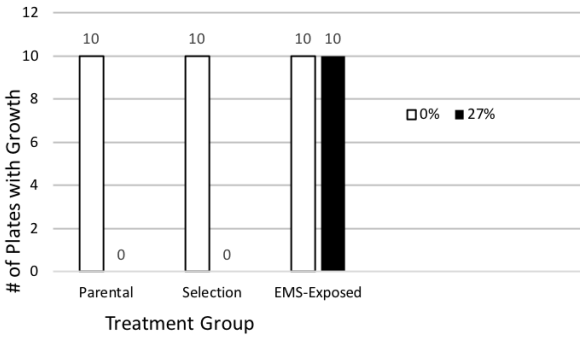


Figure 1: This figure shows how many plates each of the treatment group strains grew on at either 0% ethanol or 27% ethanol.

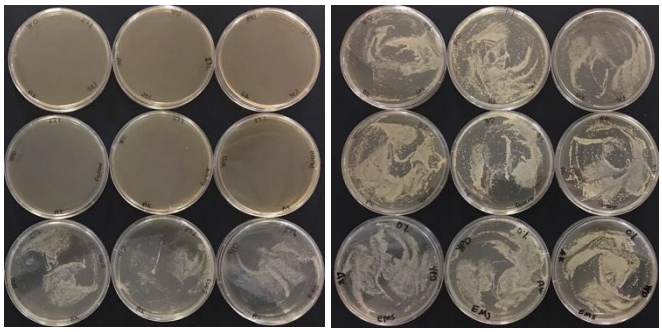


Figure 2: The image on the left shows all three strains at 27% ethanol (Top: selection, Middle: parent, Bottom: EMS-exposed) with only the EMS-exposed strain showing growth. The image on the right shows all three strains at 0% ethanol (Top: selection, Middle: parent, Bottom: EMS-exposed) with all three strains showing growth.

DISCUSSION

Saccharomyces cerevisiae naturally ferment sugars into ethanol, but due to ethanol toxicity, the process is not very efficient. If S. cerevisiae could tolerate higher levels of ethanol, then they could produce more of the product to be harvested for various uses. My research aimed to identify an efficient mechanism to increase the ethanol tolerance using artificial selection techniques, EMS as a chemical mutagen and increasing levels of ethanol as a stressor.

The process produced significant increases to ethanol tolerance in the new strain. However, the new strain was not tested to see precisely how much ethanol it could produce, only how much it could tolerate in its environment. Also, based on methods used to add ethanol to agar plates, it was difficult to determine final concentrations on plates after growth had taken place. There may have been various amounts of ethanol that evaporated from the plates that altered final concentrations throughout the growth period. Further research would need to be done on the strain to detect how much ethanol it could produce compared to the parental strain.

Regardless of the shortcomings of this paper, I found that the combining effects of artificial selection, ethanol as a stressor, and EMS as a chemical mutagen may be an effective method for increasing tolerance in *Saccharomyces cerevisiae.*

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