ISOLATION AND CHARACTERIZATION OF HIGH STRESS TOLERANCE YEAST FROM VARIOUS SOURCES IN BANGLADESH

By

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A thesis submitted to the Department of Mathematics and Natural Sciences in partial fulfillment of the requirements for the degree of
Bachelor in Microbiology

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Declaration

It is hereby declared that

1. The thesis submitted is my own original work while completing degree at BRAC University.
2. The thesis does not contain material previously published or written by a third party, except where this is appropriately cited through full and accurate referencing.
3. The thesis does not contain material which has been accepted, or submitted, for any other degree or diploma at a university or other institution.
4. I have acknowledged all main sources of help.

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Abstract

Bioethanol is becoming popular day by day because of its increasing demand as a renewable resource. A single celled eukaryotic living organism called yeast plays a vital role in producing alcohol. Though there are many organisms can interfere the process like acetobacter. However, it is almost impossible for other organism to compete with yeast due to its instinct. In addition, yeast releases alcohol as a byproduct which has strong antimicrobial activity. Thus, yeast is dominant in its environment since most of the microorganisms cannot tolerate alcohol. on the contrary, increasing amount of alcohol can also inhibit the growth of yeast and put the organism in stationary phase. The aim of this study is to isolate and characterize a yeast which can tolerate high concentration of alcohol as well as stress and can produce more alcohol before reaching the stationary phase. For this purpose, primary sample has been collected from Lavender flower, Apple skin, Grape skin, honey and yeast from stressed environment created manually. In thermo tolerance test, isolated yeast sample showed significant growth at 27˚C and 30˚C. on the other hand, it showed high osmo tolerance in addition of NaCl in different concentration. Lastly, it showed significant growth in 5% and 10% ethanol concentration. Since it showed a significant tolerance in the tests, it could be a promising strain to use in future for industrial purpose.

Keywords: Bioethanol; Yeast; Alcohol tolerance; Stress tolerance; Thermo tolerance; Osmo tolerance

"This is dedicated to my family and loved one's who have supported and encouraged me unconditionally"

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List of Acronyms

YPD	Yeast Peptone Dextrose
ml	Milliliter
g/l	Gram per liter
OD	Optical Density

Chapter 1

Introduction

Most of the refined fuel come from petroleum has been found beneath the earth surface. Due to urbanization, the resources are decreasing gradually. However, the demand of renewable energy source is also increasing. Thus, the attention towards renewable source of energy like ethanol is increasing because of the decreasing petroleum resources (Nadir et al., 2009). Moreover, toxicity of ethanol is lower than that of other fossil fuels like diesel, petroleum etc. In addition, combustion of ethanol reduces the amount of volatile organic compounds like carbon monoxide and nitrogen oxides (Charles E. Wyman & Norman D. Hinman, 2016).

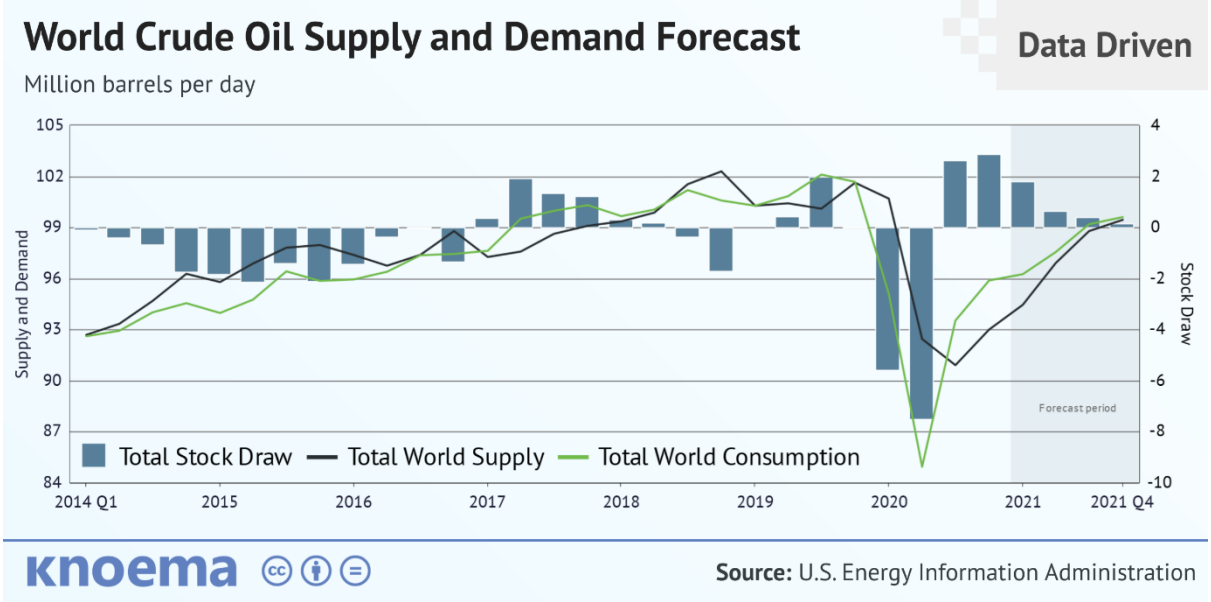

Figure 1: World crude oil supply and demand forecast

According to US Energy information administration, the total global oil supply in 2021 is about 93.39 million barrels per day however, the demand is 95.19 million barrels per day. So, the demand is increasing day by day whereas, the oil production has decreased. In this case we

should focus on the production of biofuel which is ecofriendly as well as more efficient. (Figure: 1)

Yeast *Saccharomyces cerevisiae* has been genetically engineered to produce biofuels. Some of them are, 1-butanol and isobutanol which is a substitute for gasoline. Yeasts are also used in the production of jet fuel called amorphadiene. *Saccharomyces cerevisiae* has become one of the most promising yeast strains to produce biofuel efficiently for future (Buijs et al., 2013; Uroš Petrovič, 2015).

The purpose of this study was to isolate an efficient yeast which has high alcohol and stress tolerance. It indicates that the isolated yeast would be able to survive in potentially high alcohol concentration and extreme stress like osmotic pressure, high temperature and increased concentration of carbohydrate. Thus, it can produce alcohol efficiently in any condition as well as increase the production. For this study, sample were collected from lavender flower (L), grape skin (G), apple skin (A), baker's yeast (B), pure honey (H).

Chapter 2

2.1 Materials and methodology

Fermentation is a natural process that is widely used in making alcoholic beverages. In this process, microorganisms convert carbohydrates into alcohol or acid. Usually, yeasts convert sugars into alcohol and CO_2 in the fermentation process (Maicas, 2020).

There is a link between alcohol tolerance of yest and CO_2 production. If a yeast strain has higher alcohol tolerance it can go further in fermentation process result in increased CO_2 production. So, CO_2 production can be an indicator of alcohol tolerance (Gaden, 2000; Maicas, 2020). Figure: 2 has been adopted from (Faria-Oliveira et al., 2013)

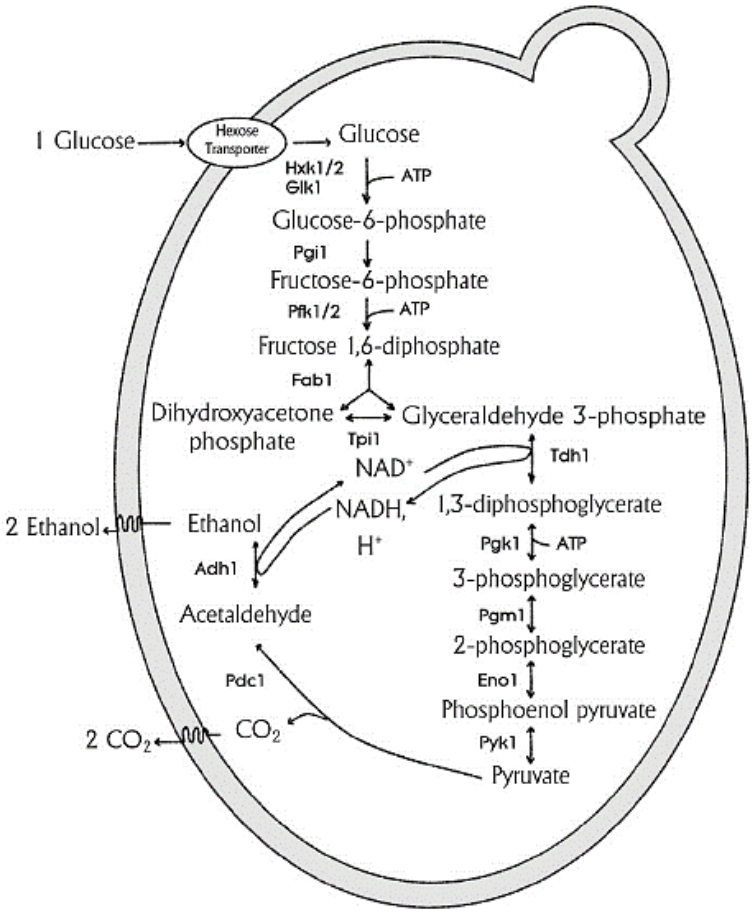

Figure 2:Alcoholic fermentation - enzymatic steps on S. cerevisiae

2.2 Sample collection

Yeast sample was collected from Gulshan 1 nursery; Mohakhali fruits store, Dhaka; pure mustard flower honey was collected from Jamalpur, Bangladesh and baker's yeast from 'Saf' brand. The samples were kept in autoclaved water and incubated at 30˚C for 48 hours. After incubation, samples were kept in the refrigerator for further study.

Figure 3: sample collection

2.3 Materials and media

Durham tube, small conical flask, test tube, autoclave, microscope, spectrophotometer has used to perform the study. Moreover, YPD media and YPD broth was used which contained: agar 15g/l; peptone 20g/l; yeast extract 10g/l, glucose/fructose 20g/l (Sitanggang et al., 2010; Wei et al., 2013).

2.4 Inducing stress

Batch Fermentation was performed with pure cherry grape juice by baker's yeast. Then fermentation was carried out for about five months. However, production of gas was greatly reduced after 3 months. The fermentation was carried out for an extra five months so that the yeast can adapt to stress. (Figure: 3)

Firstly, the sample was collected from the fermenter. Secondly, collected sample was cultured in YPD media to isolate probable yeast colonies primarily by observing colony morphology.

Thirdly, microscopic examination has performed for the final verification of yeast. Finally, several stress tolerances tests have been done to identify the most efficient yeast that has high ethanol and stress tolerance. Yeasts were initially identified by visual appearance on microscope. Yeast used in this study were in cluster form in according to microscopic appearance (Knop, 2011; Vopálenská et al., 2005).

Figure 4: Fermented Grape Juice

2.5 Yeast isolation method

The samples were inoculated in YPD broth and incubated at 30˚C for 48 hours (Iticha, 2016; Sakamoto et al., 2019). From five types of sample, primarily lavender was used for the characterization of yeast in this test because of its higher gas production during primary fermentation. After visual observation, sample from this test tube was diluted up to 10^{-5} and plated on YPD culture media and again incubated at 30˚C for 48 hours. Then yeast colonies were identified based on their colony morphology, colony pigment, surface appearance and edge.

2.6 Microscopy analysis

Microscopic observation was performed for the confirmation of the yeast. Samples were taken by a cotton swab to introduce in the microscopic plate mixed with sterile water. Then the plate was left until dry out. Then the samples were observed under the microscope at 60x and 100x.

2.7 Determining stress tolerance yeast

Several stress tolerances tests have done to identify the most reproductive yeast which can survive in high carbohydrate concentration, higher concentration of alcohol, unfavorable temperature and high osmotic pressure. The samples were grown in YPD broth before performing each of the test.

2.8 Thermo tolerance test

In this test, yeast cultures were inoculated into four different test tubes containing YPD broth and kept for incubation at 27˚C**,** 30˚C, 37˚C and 45˚C for 48 hours. Initial OD was taken from each test tube using spectrophotometer at 600nm (Negera, 2017). OD will increase gradually with the increased cell mass (Gray, 1941). After incubation, final OD (600nm) was taken from each of the test tube.

2.9 Osmo tolerance test

Yeast sample was inoculated into five different test tubes containing YPD broth added with 5%, 10%, 15%, 20% and 25% of NaCl. Then, the test tubes were incubated at 27˚C for 48 hours. OD was taken before and after incubation for the confirmation of result. As the thermo tolerance test showed best growth at 27 ˚C, further studies were done at this temperature.

2.10 Ethanol tolerance test

Initially, five different test tube containing YPD broth was prepared with alcohol concentration of 5%, 10%, 15%, 20% and 25%. After that yeast was inoculated and kept at 27˚C for 48 hours(Iticha, 2016). OD was taken for the confirmation of growth.

2.11 Carbohydrate concentration tolerance test

For this test, five different concentration (5%, 10%, 15%, 20% and 25%) of carbohydrate (glucose) was prepared with YPD broth. After that, yeast was inoculated and kept at 27˚C for 48 hours(Iticha, 2016)

Chapter 3

Result and discussion

3.1 Primary Fermentation

Firstly, primary fermentation carried out by inoculating five different samples into five different conical flasks containing YPD broth. The flasks were then incubated at 30˚C for 48 hours. After incubation gas production has been observed. From the fermentation pathway, we can see that, after consuming one molecule of glucose, one yeast cell is releasing two molecules of ethanol and two molecules of CO_2 as a byproduct. (Figure: 2) Thus, more the carbohydrate consumption, more ethanol and CO_2 will be produced. So, gas production is a clear passive indicator of alcohol production (Ronnie Willaert, 2008).

Figure 5: Fermentation of sample (L, G, A, B, H)

3.2 Isolation and identification of yeast sample

From the primary fermenter the sample (L) was isolated using YPD culture media by serial dilution method (Figure: 6). For the identification of the yeast, several steps were done. Firstly, colony morphology was observed and then microscopic observation was done by standard

protocol (Figure:6). In the microscopic view, yeasts were clustered together like budding yeast and pulvinate shape in culture plate. (Figure:7)

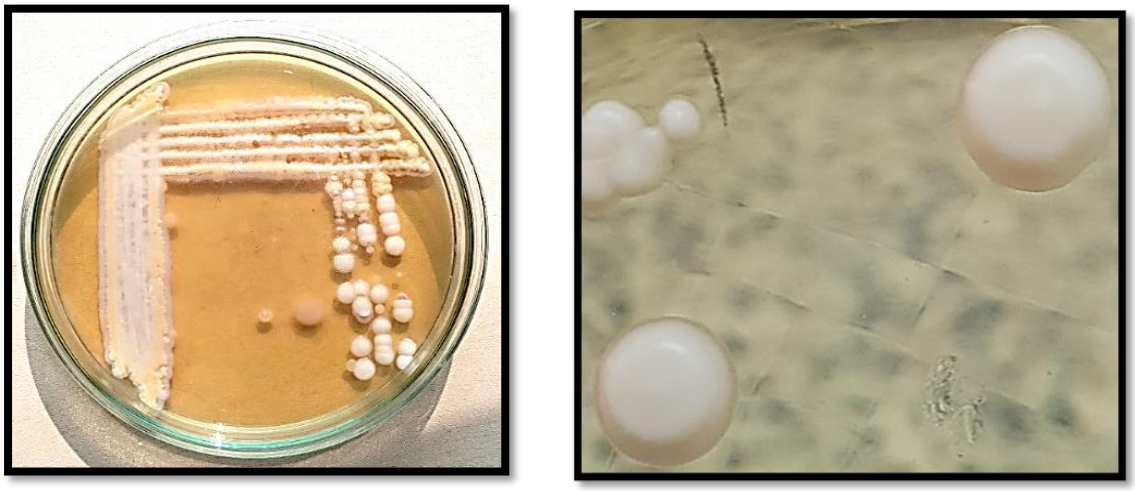

Figure 3: Yeast culture

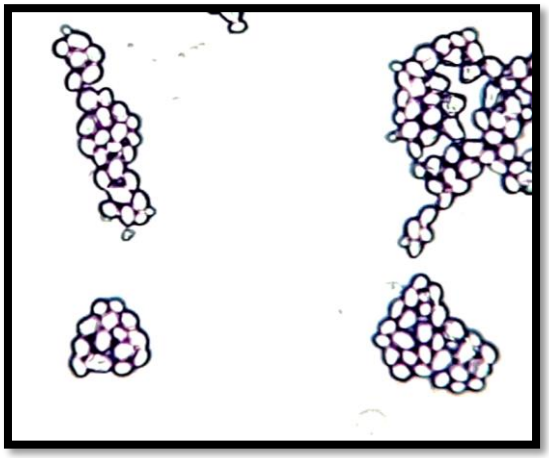

Figure 4:Microscopic view of Yeast culture

3.3 Thermo tolerance test

Yeast samples were inoculated into YPD culture media for the thermo tolerance test and kept in different temperature. After the incubation for 48 hours, yeast sample that showed significant growth in 27˚C and 30˚C. In 37˚C and 45˚C, it showed moderate growth. This result indicated that the yeast sample was able to grow in higher temperature. To confirm the result this test was done in liquid media and OD (600nm) was taken for the confirmation of the growth.

Comparing the OD result taken in 0 hour and 48 hours, there was significant growth at 27 ˚C. However, the yeast was also able to grow at 45 ˚C means it has higher temperature tolerance. (Figure: 8; Table: 1)

Temperature	Initial OD (0hr)	Final OD (48hr)
27˚C	0.329	1.555
30˚C	0.225	1.190
37˚C	0.311	0.540
45˚C	0.229	0.165

Table 1: Thermo Tolerance

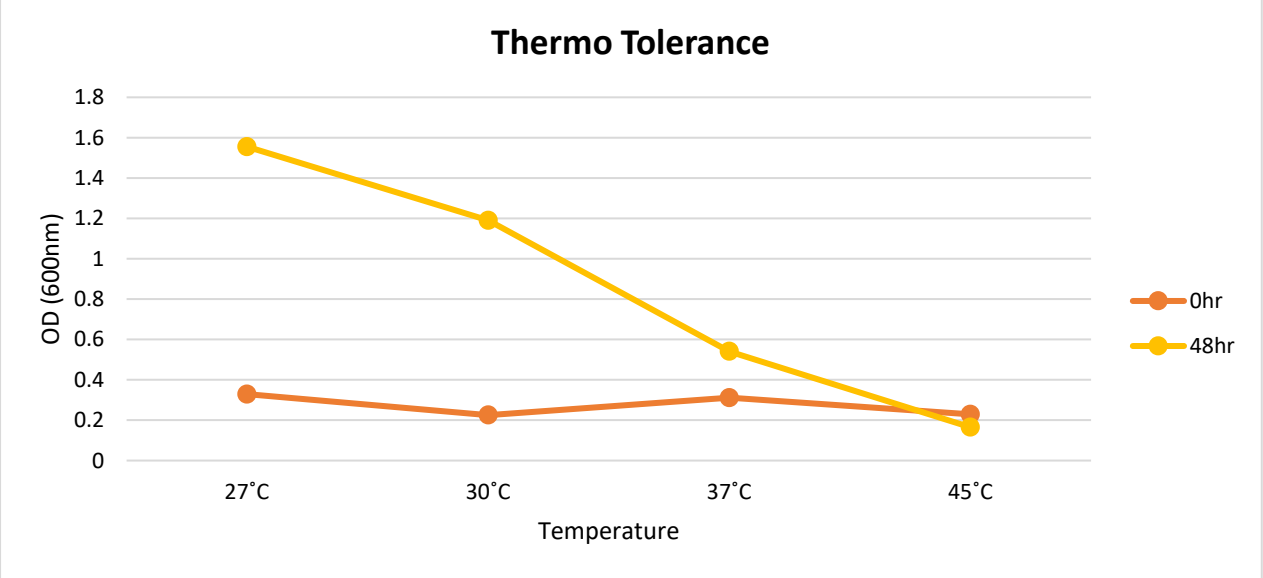

Figure 5: Thermo Tolerance

3.4 Osmo tolerance test

For osmo tolerance, NaCl of different concentration was used. This test was done in YPD broth. After inoculation, media containing the yeast samples were kept at 27˚C for 48 hours and OD was taken in 0 hour as well as in 48 hours of incubation. OD results showed that there was significant growth in 5-10% NaCl concentration. Moreover, yeast sample was able to grow

moderately in 15%, 20%, 25% of NaCl concentration which proves that the yeast could tolerate and survive in high osmotic pressure. (Figure: 9; Table: 2)

NaCl percentage	Initial OD (0hr)	Final OD (48hr)
5%	0.214	0.901
10%	0.231	0.441
15%	0.249	0.237
20%	0.201	0.165
25%	0.198	0.128

Table 2: Osmo tolerance

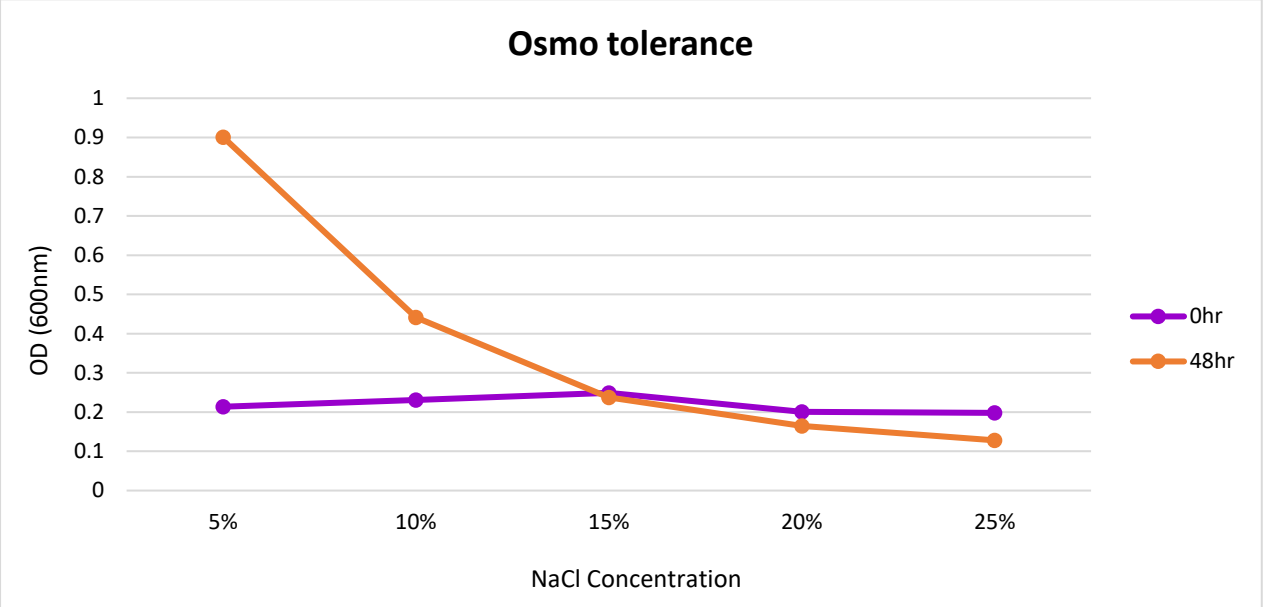

Figure 6: Osmo tolerance

3.5 Ethanol tolerance test

Ethanol tolerance test was done for five different concentration which were 5%, 10%, 15%, 20% and 25%. This test was done in YPD broth and it was kept at 27˚C, 48 hours for incubation. OD was taken in 0 hour and 48 hours for the confirmation of result. The result shows best growth at 10% concentration of ethanol. Meanwhile, growth has also been observed at 25% concentration of ethanol. (Figure: 10; Table: 3)

Ethanol percentage	Initial OD (0hr)	Final OD (48hr)
5%	0.299	1.801
10%	0.301	1.521
15%	0.251	0.607
20%	0.216	0.225
25%	0.204	0.201

Table 3: Ethanol tolerance

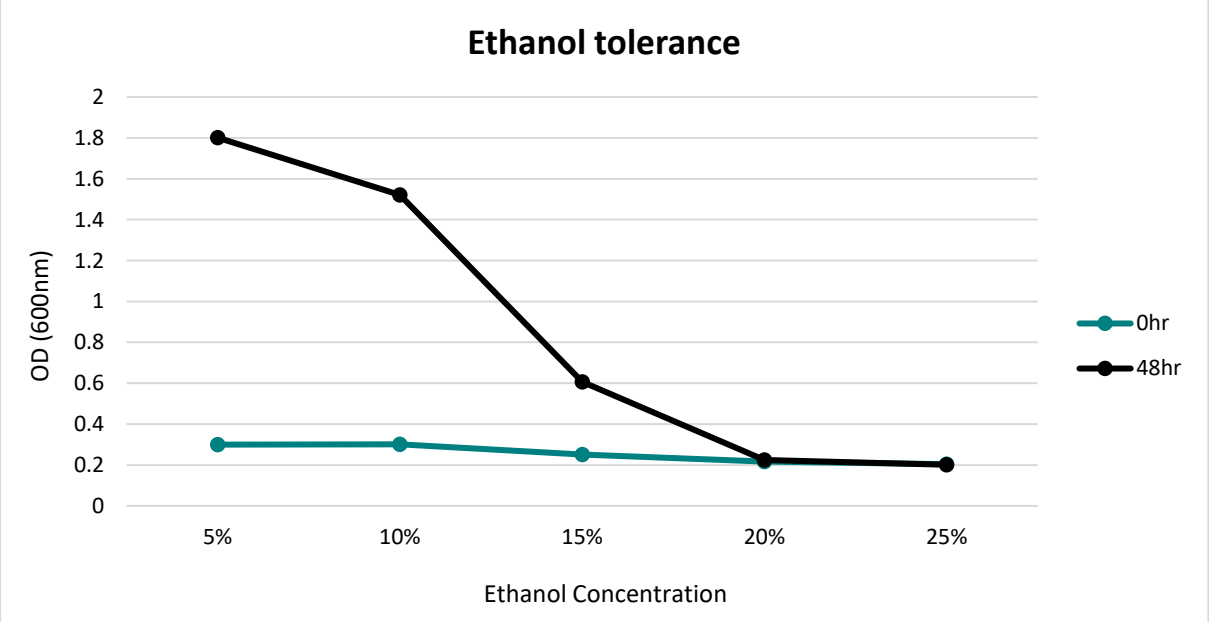

Figure 7: Ethanol tolerance

3.6 Carbohydrate concentration tolerance test

In this test, 5%, 10%, 15%, 20%, 25% glucose was used as a source of carbohydrate. Yeast sample was inoculated into the media and kept at 27˚C for 48 hours to observe. After 24hr incubation, there was gas formation which indicated that the yeast was able to ferment glucose. Unfortunately, due to corona virus pandemic it was not possible to take the OD in 48hr to confirm the growth. For this instance, this will be studied further in future. (Figure: 11; Table:4)

5% 10% 15% 20% 25%

Figure 8: Carbohydrate tolerance (glucose)

Concentration of sugars	Production of gas
5%	Yes
10%	Yes
15%	Yes
20%	Yes
25%	Yes

Table 4: Carbohydrate concentration tolerance

Chapter 4

Conclusion

In short, yeast found in this study showed significant tolerance in the tests that were done. In thermo tolerance test, it showed highest growth in 27˚C. As for the carbohydrate test, there was visible gas formation indicating that the yeast can survive higher carbohydrate concentration. Sequentially, in osmo tolerance test, 5% and 10% NaCl concentration showed significant growth. In addition, it showed a notable growth in 5% and 10% ethanol concentration test. Unfortunately, other remaining tests were not possible to do for the pandemic. As primary fermentation showed significant gas production for sample collected from Lavender rather than other samples. This yeast has higher probability of having more stress tolerance than others. Further studies should have to be done on other samples as well.

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