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*Research Article*

# Development of Alcohol-Tolerant Yeast Strains and Assessment of their Fermentation Efficiency

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**ABSTRACT**

*Ethanol consumption rises with urbanization as people seek alternative fuels, industrial solvents, cleaning agents and preservatives. The synthesis of ethanol by Saccharomyces cerevisiae is influenced by a number of intrinsic variables, including fermentation conditions such as temperature, pH, ethanol concentration and sugar concentration. Ethanol is the main result of yeast sugar fermentation under anaerobic conditions. On the other hand, ethanol may inhibit the activity of yeast cells like yeast growth, viability, and fermentation rates. The current study focuses on developing alcohol-tolerant strains by inducing alcohol stress. The developed alcohol-tolerant strains are studied for their morphological and biochemical characteristics and evaluated for their fermentation efficiency. The testing results revealed that all the produced alcohol-tolerant strains were able to grow well at alcohol concentrations of 10%, 12%, 14%, 16%, 18%, and 20%. The examination of developed alcohol-tolerant strains revealed that all the strains are of Saccharomyces cerevisiae and are able to ferment sucrose to ethanol. The fermentation efficiency of control yeasts ($C_M$ and $C_Y$) was determined to be 93.7%. Alcohol-tolerant strains with 18% and 20% alcohol strength demonstrated fermentation efficiency ranging from 88% to 91%. The alcohol-tolerant strains $M_4$ – molasses-based culture and $Y_4$– YPD-based culture with 16% alcohol strength achieved the highest fermentation efficiency of 98.2% and 98.3%, respectively. The alcohol-tolerant yeast strains - $M_4$ and $Y_4$ could serve as potential strains for fermentation even under high ethanol concentrations and could be used at the industrial level for fermentation of various raw materials in order to increase bioethanol output.*

***Keywords:*** *Distillery; Ethanol; Molasses; YPD medium and Alcohol Tolerance.*

## INTRODUCTION

Microscopic organisms that can exist as single cells, multicellular animals, or clusters of cells are known as microorganisms or microbes. They can be bacteria, archaea, protozoa, algae, fungi or viruses that are abundant in nature and helpful to life, but some can be extremely harmful.

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These microorganisms have both beneficial and detrimental effects on the food industry, distillery, health sector, and agriculture, among other industries. The distillery sector primarily produces a drinkable liquid that contains ethanol from the distillation of fermented grains, fruits, or vegetables; thus, it is called a distilled beverage, spirit, or spirit. In India, there are roughly 295 distilleries, most of which are located in the states of Tamil Nadu, Gujarat, Maharashtra, Uttar Pradesh, Andhra Pradesh, and Karnataka (Tyagi et al., 2010). Commercial alcohol, industrial chemicals, and biofuels are among the uses for which ethanol is produced. In the distillery industries, sugarcane molasses or other readily available sugar sources are treated in the presence of microbes to produce mild alcoholic beverages through a fermentation process with distillation involved. The synthesis of renewable chemicals as alternative energy sources can be significantly replaced by the fermentation of ethanol, which utilizes renewable resources in addition to its use. As ethanol is similar to petrol fuel in many ways, it is really being extensively researched as a renewable fuel source (Jones & Ingledew, 1993).

Yeast is the organism used to generate food, wine, beer, and various biochemicals, including bioethanol. Their easy genetic manipulation and rapid replication make them popular model organisms. Yeast has been thoroughly investigated for its genome and organization, and it has a doubling time of ninety minutes **(**Martini, 1992). A yeast strain's suitability for industrial use depends on a few physiological traits. Some essential properties for use in industrial ethanol production are ethanol tolerance, sugar tolerance, and invertase activity (Jiménez et al., 1986). Yeast strains have been chosen for effective ethanol synthesis, and molasses, a byproduct of the sugarcane or sugar beet processing industries, is frequently utilized as a raw material for ethanol production for financial reasons. On average, *Saccharomyces cerevisiae* yeast strains are utilized to manufacture bioethanol under closely watched optimization conditions, allowing for the bioconversion of substrate to increase bioethanol yield. *Saccharomyces cerevisiae*, often known as baker's yeast or brewer's yeast, is frequently utilized in the manufacturing of ethanol because of its capacity to convert carbohydrates into ethanol and $CO_2$. The anaerobic fermentation of several sugar sources by *Saccharomyces cerevisiae* yields about 80% ethanol (Bai et al., 2008; Rolz et al., 2011). A wider variety of sugars, including those originating from lignocellulosic biomass, can be utilized by *S. cerevisiae* strains that have undergone genetic engineering to enhance fermentation efficiency and boost ethanol tolerance. To maximize ethanol yield, minimize production costs, and have the least negative environmental impact possible, it is imperative to select the right strain of *S. cerevisiae* and optimize fermentation conditions.

There are various feedstocks from which bioethanol can be made, such as starchy, sugary, and cellulose materials. Corn, sugar cane, bagasse, sugar beets, sorghum, switch grass, barley, hemp, potatoes, sunflower, wheat, wood, paper, straw and cotton are an assortment of feedstocks used in biomass production. The main raw material used to produce ethanol is sugar cane, either as cane juice or as molasses, a byproduct of sugar mills. However, sugar cane molasses is used instead of cane juice as the main raw material in India. For the manufacturing of ethanol, fermentable sugars can also be obtained from beetroot molasses. Molasses is the noncrystalllizable residue left over after sucrose purification. It has several benefits, including being a readily available, reasonably priced raw material that does not require starch hydrolysis and has already been used to produce ethanol. Molasses obtained from sugar beet processing contains about 60% sucrose and 40% other substances, including inorganic salts, raffinose, ketose, organic acids, and compounds containing nitrogen (Belitz et al., 2009, & Satyanarayana et al., 2009). Ethanol generates energy that is renewable and less carbon intensive than crude

oil. Bioethanol reduces air pollution and greenhouse gas emissions (GHG) to help slow down climate change because it emits cleaner emissions. Furthermore, ethanol made from sugarcane lowers greenhouse gas emissions by 86 to 90% in the absence of a major change in land use, according to multiple studies (Rajagopal & Zilberman, 2008).

The synthesis of bioethanol is influenced by numerous factors. Along with the fermentation parameters of temperature, pH, ethanol concentration, and sugar concentration, other intrinsic variables affecting ethanol synthesis include immobilization, dissolved oxygen, culture medium, and other micronutrients. Additionally, the medium regulates the specific rate of fermentation, the absorption of nutrients, and the viability of the yeast. The effect of these factors directly or indirectly inhibits the growth of yeast, resulting in a reduction of the production of bioethanol. Various stimuli cause different kinds of stress on yeast and the fermentation process, and their target sites vary as well. One of the main factors influencing yeast growth and alcohol production is the concentration of ethanol. The main product of yeast sugar fermentation is ethanol. However, ethanol is highly harmful to yeast cells and other microbes at higher concentrations. Ethanol produced during fermentation or when supplied externally has a complicated inhibitory impact. It has been demonstrated that ethanol affects the yeast population's specific rates of fermentation, viability, and growth in distinct and separate ways. While strong fermentative capability was only reduced at higher ethanol concentrations, inhibition of cell growth and viability was found to increase with increasing ethanol concentrations.

Ethanol stops yeast from growing at relatively low concentrations by decreasing cell volume, preventing cell division, and raising specific growth rate. Conversely, high ethanol concentrations reduce cell viability and cause cell death. Additionally, ethanol has unique effects on the development, viability, and particular rates of fermentation of the yeast population. By causing the synthesis of heat shock proteins, slowing down the rate at which proteins and RNA accumulate, increasing the frequency of small mutations, altering metabolism, denaturing intracellular proteins, and lowering the activity of glycolytic enzymes, ethanol has an impact on macromolecular biosynthesis and cell metabolism (Hu et al., 2007). Ethanol target to affect and damage the membranes of different cellular organelles and the plasma membranes in yeast and in other microbes. Ethanol damages the cell membrane, which changes the permeability and organization of the membrane. It has been observed that the inhibitory effects of alcohols increase with increasing carbon number, providing evidence that the potency of alcohols is connected with their lipid solubility (D'Amore et al., 1990). Numerous more mechanisms have been put up to explain ethanol's inhibitory effects. These include the following: inhibition of glucose, maltose, ammonium, and amino acid transport; depression of the optimum and maximum temperature for growth; enhancement of thermal death and "petite" mutation in yeast; and inhibition of glucose-induced proton accelerated passive re-entry of protons in a manner resembling the action of an uncoupler. Additionally, it was shown that the inhibitory effects of alcohols increased with carbon number, indicating a possible relationship between alcohol potency and lipid solubility. Ethanol tolerance is particularly important since it is nearly hard to avoid during fermentation, even though other factors, such as substrate inhibition, can be avoided by introducing substrate gradually. The increasing demand for ethanol for a range of industrial applications, such as industrial solvents, cleaning agents, preservatives, and alternative energy sources, is forcing ethanol output to increase.

To cope with the increasing demand for alcohol, ethanol production from various feed stocks is being encouraged. The major problem encountered during the production of alcohol from feedstocks with high sugar concentrations, like ethanol from grains, is the

effect of feedback inhibition by the ethanol produced. The way out of this problem may be the development of alcohol-tolerant yeast strains.

## MATERIAL AND METHODS

### Sample Collection

B Heavy Molasses sample was obtained from Experimental Sugar Factory, National Sugar Institute, Kanpur, Uttar Pradesh, India.

### Primary Analysis and Fermentation

The molasses sample was analyzed using criteria like pH, Brix, Total Reducing Sugar (TRS), Reducing Sugar (RS), Sludge, Total Dissolved Solids, and Specific Gravity. It was diluted to 10% TRS and analyzed for various parameters. The diluted molasses sample was allowed to ferment both aerobically and anaerobically for 24 hours at 30°C. Commercial yeast, urea, and sodium phosphate dibasic were added as supplements.

### Growth on Medium

The Molasses Medium and YPD Broth Medium (Yeast Extract: 10g, Peptone: 20g, Dextrose: 2g, and Distilled Water: 1000 ml) with different alcohol strengths (10%, 12%, 14%, 16%, 18% and 20%) were inoculated with a pre-grown yeast culture from an aerobic flask. The pH range for both media was adjusted to 4.5–5.0. For 24 hours, all the 14 flasks were incubated at 30℃. After this, cell viability was determined for each sample.

### Growth on YPDA Medium

The yeast cultures grown on Molasses Medium and YPD Broth Medium, which had different strengths of alcohol, were isolated in YPDA Medium by streak plate method.

### Pure Culture of yeasts

Pure cultures of the isolates from Molasses Medium and YPD Broth Medium grown on YPDA Medium Were obtained and maintained in slants.

### Identification and Characterization of yeasts

The yeast isolates were identified based on their macroscopic, microscopic and biochemical properties. The colonies isolated on YPDA media were studied for morphological characteristics on the basis of colour, texture, margin, shape, elevation and colony size. Lactophenol Cotton Blue Staining was done to observe yeast cells' shape and bud formation. The Pellicle Formation Test was done by inoculating the yeast cultures in YPD Broth Medium and incubating it for 48 hrs. The Carbohydrate Fermentation Test was performed to find out the ability of the isolated yeast cultures to ferment specific carbohydrates by inoculating cultures of yeast in a test tube containing basal medium and Durham's tube with indicator methyl red (Taye Negera, 2017). The test was carried outusing nine different carbohydrate sources- Dextrose, Starch, Fructose, Sucrose, Galactose, Trehalose, α-Methylglucoside, Mannitol and Maltose.

### Distillation and determination of Fermentation Efficiency

Yeast cultures from YPDA slants of molasses and YPD Broth Medium were transferred to molasses medium for aerobic fermentation at 30℃ for 24 hours. After 24 hours, cultures from aerobic flasks of Molasses and YPD Broth Medium were transferred to Molasses Medium (100ml each) for anaerobic fermentation at 30℃ for 24 hours. After fermentation distillation was done for each yeast isolate, the strength of the alcohol was evaluated, and fermentation efficiency was estimated.

## RESULT AND DISCUSSION

### Primary Analysis of the Molasses

B Heavy Molasses sample was taken from the National Sugar Institute, Kanpur, and analyzed for various parameters. The results of the analysis are given in Table 1. The Total Reducing Sugar, Reducing Sugar, Specific Gravity, Sludge, Brix, pH, and Total Dissolved Solid of the B Heavy Molasses sample were found to be 54.4%, 2.8%, 1.42, 11.7%, 84˚, 4.8, and 35.2 PPT, respectively. The molasses obtained from NSI was diluted to 10% for further study.

### Aerobic and Anaerobic Fermentation

For the purpose of aerobic and anaerobic fermentation, the molasses having the TRS 54.4% was diluted as per the formula

N1V1=N2V2 to 10%, and various parameters were analyzed for the same. The result of the analysis carried out is given in Table 1.

The diluted Molasses sample's Total, Reducing Sugar, Specific Gravity, Sludge, Brix, pH and Total Dissolved Solid were 10.25%, 0.3%, 1.052, 8.4%, 28˚, 4.8 and 3.37PPT respectively. The molasses sample was subjected to aerobic and anaerobic fermentation. Following the aerobic fermentation of molasses by yeast, the number of yeast cells grown was counted using a Hemocytometer (Table 2), and the anaerobically fermented molasses was subjected to distillation to determine the percentage of alcohol.

Following aerobic fermentation for 24 Hrs at 30˚C, Total Cell Count was found to be $390\times10^6$Cells/ml, whereas Dead Cells, Viable Cells and Bud Count were found to be 1.28%, 98.71% and 6.41%, respectively. The percentage of alcohol distilled from anaerobically fermented molasses was found to be 5.6%.

**Growing of Yeast on Molasses Medium and YPD Broth Medium**

Both Molasses and YPD Broth Medium were prepared and taken in different flasks marked as $C_M$ (Control), $M_1$(10%), $M_2$ (12%), $M_3$ (14%), $M_4$ (16%), $M_5$ (18%), and $M_6$ (20%) and $C_Y$ (Control), $Y_1$ (10%), $Y_2$ (12%), $Y_3$ (14%), $Y_4$ (16%), $Y_5$ (18%) and $Y_6$ (20%) respectively. Alcohol of different concentrations (10%, 12%, 14%, 16%, 18%, 20%) was added to the respective flasks with molasses, and YPD medium and alcohol was not added in the Control flasks ($C_M$ and $C_Y$). This is followed by inoculation of yeast culture from aerobically fermented culture.

**Table 1: Analysis of B Heavy Molasses and Diluted Molasses Sample**

| PARAMETERS | B Heavy Molasses | Diluted Molasses (10%) |
|---|---|---|
| | Values Obtained | Values Obtained |
| TRS (%) | 54.4 ± 0.2 | 10.25 ± 0.03 |
| RS (%) | 2.8 ± 0.1 | 0.03 ± 0.02 |
| Specific Gravity | 1.42 ± 0.01 | 1.052 ± 0.001 |
| Sludge (%) | 11.7 ± 0.2 | 8.4 ± 0.2 |
| Brix (˚) | 84 ± 1 | 28 ± 1 |
| pH | 4.8 ± 0.1 | 4.8 ± 0 |
| TDS (PPT) | 35.2 ± 0.2 | 3.37 ± 0.02 |
| Alcohol (%) | - | 5.6 ± 0.02 |

The growth of yeast was observed in control and alcohol treated Molasses Medium and YPD Broth Medium of different strength (10%, 12%, 14%, 16%, 18% and 20%) after 24 Hrs of incubation at 30˚C.

Seven yeast cultures isolated from local fermented foods, which were named F01, F02, F08a, F08b, F08c, F10, and F13, showed successful growth at a concentration of 16% ethanol. Only isolate F08b grew well at a concentration of 17% ethanol. Meanwhile, isolates F01, F08a, F10, and F13 grew at an average rate, while isolates F02 and F13 continued to develop, albeit slowly. The majority of the yeast isolates were unable to grow at 18% ethanol concentrations. Only isolate F01 survived and grew at high ethanol concentrations (Nurcholis et al., 2021).

**Table 2: Cell Counting of Molasses Medium from Aerobic Fermentation**

| Cell Counting | |
|---|---|
| Total Cell Count (Cells/ml) | $390\times10^6$ |
| Bud Count (%) | 6.41 |
| Viable Cells (%) | 98.71 |
| Dead Cells (%) | 1.28 |

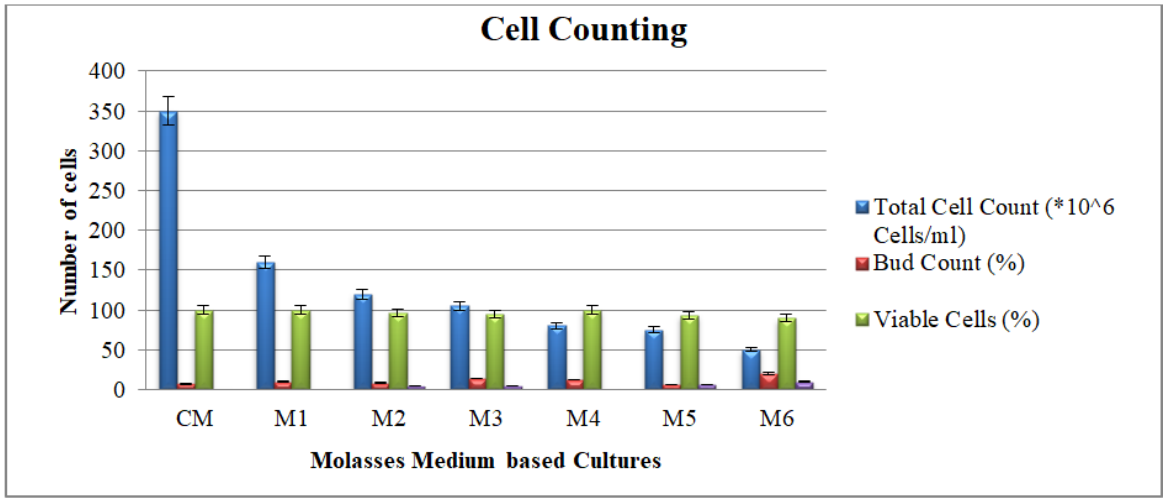


**Figure 1: Counting of Yeast Cells grown in Molasses Medium with different strength of alcohol**

**Determination of Cell Viability**

A hemocytometer was used to assess the viability of the cells in all the inoculated flasks following a 24-hour incubation period.

The total number of cells, viable cells, dead cells, and budding count were counted, and the percentage calculated was given in Figure 1 and Figure 2 for cultures grown in Molasses and YPD medium with different alcohol concentrations. The cell counting was found to be high in the control ($C_M$ and $C_Y$) that was not treated with alcohol. The total cell counting was found to be decreasing with an increase in the alcohol stress. The dead cells were not found in the case of control ($C_M$ and $C_Y$) and 10% alcohol ($M_1$ and $Y_1$), whereas with increasing the alcohol percentage, the dead cells also increased due to the stress created by the alcohol. The viability of the cells decreases with an increase in alcohol concentration. The bud count percentage was found to be higher with an increase in alcohol concentration. The maximum percentage of bud count was found to be in $M_6$ and $Y_6$, which was treated with 20% alcohol.

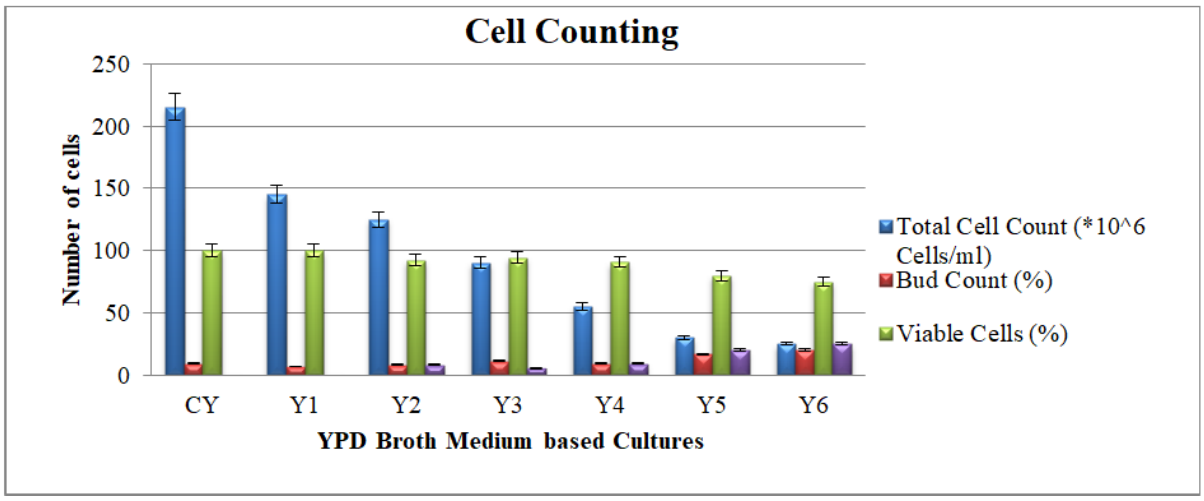


**Figure 2: Counting of Yeast Cells grown in YPD Broth Medium with different strength of alcohol**

**Isolation of Alcohol Tolerant Strainson YPDA Medium**

The yeast cultures grown on Molasses and YPD Broth Mediums with different strengths of alcohol were isolated in YPDA Medium by streak plate method. Isolated yeast from different alcohol concentration mediums is shown in Figures 3-4.

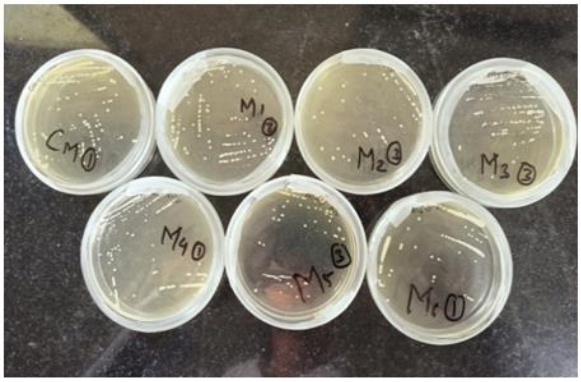

**Figure 3: Growth of Yeast Cultures from Molasses Medium on YPDA Medium**

**Figure 4: Growth of Yeast Cultures from YPD Broth Medium on YPDA Medium**

Yeast cultures isolated from fruits like pineapples developed butyrous and smooth white elevated colonies (Naser, 2014). Isolated yeast from sugarcane juice showed colonies with smooth surfaces, round edges, and colours ranging from cream to white (Yadav & Tiwari, 2016). The yeast isolated from food sources such as yogurt, mango juice, buttermilk, etc. showed smooth-surfaced, raised, cream-colored, and soft colonies (Khattab et al., 2016). The individual colonies from the plates were used further for pure culture preparation.

**Pure Culture of the Alcohol Tolerant Yeast Isolates**

Pure Culture was prepared by picking single isolated colonies obtained on petriplates of Molasses Medium based and YPD Broth based cultures and streaking the same on YPDA slants as shown in Figure 5 and Figure 6. The growth of pure culture was observed after 72hrs of incubation at 30˚C.

**Figure 5: Pure Culture of Alcohol Tolerant Yeast Strains from Molasses Medium Based Culture**

**Figure 6: Pure Culture of Alcohol Tolerant Yeast Strains from YPD Broth Medium Based Culture**

**Identification and Characterization of the Yeast Isolates**

The yeast isolates from plates were examined for colour, texture, margin, shape, elevation, and colony size, as indicated in Tables 3 and 4.

**Table 3: Macroscopic characteristics of Alcohol Tolerant Yeast Strains from Molasses Medium Based Culture**

| Sample | Colour | Texture | Margin | Shape | Elevation | Colony size (in mm) |
|---|---|---|---|---|---|---|
| $C_M$ | Cream | Smooth | Entire | Round | Convex | 2×2 |
| $M_1$ | Cream | Smooth | Entire | Round | Convex | 2×1.5 |
| $M_2$ | Cream | Smooth | Entire | Round | Convex | 2×2.5 |
| $M_3$ | Cream & White | Smooth | Entire | Round | Convex | 3×2.5 |
| $M_4$ | Cream & White | Smooth | Entire | Round | Convex | 2×2 |
| $M_5$ | Cream & White | Smooth | Entire | Round | Convex | 2×2 |
| $M_6$ | Cream | Smooth | Entire | Round | Convex | 2×2 |

All the isolated alcohol tolerant strains of Molasses Medium Based Cultures were creamy in colour, with some being white in addition to creamy. The texture was observed as smooth, with an entire margin. The shape was found to be round with a convex elevation. The colony size in $C_M$, $M_1$, $M_4$, $M_5$, and $M_6$ was 2×2mm, while $M_2$ and $M_3$ were significantly larger, measuring 2×2.5mm and 3×2.5mm, respectively. The colonies of alcohol-tolerant strains developed from YPD Broth Medium Based Cultures were creamy in colour, smooth in texture, with an entire margin, round in shape and convex in elevation. The size of the colony was found to be high in $Y_4$, $Y_5$ and $Y_6$ compared to that of $C_Y$, $Y_1$, $Y_2$ and $Y_3$.

**Table 4: Macroscopic characteristics of Alcohol Tolerant Yeast Strains from YPD Broth Medium Based Culture**

| Sample | Colour | Texture | Margin | Shape | Elevation | Colony size (in mm) |
|---|---|---|---|---|---|---|
| $C_Y$ | Cream | Smooth | Entire | Round | Convex | 2.5×2 |
| $Y_1$ | Cream | Smooth | Entire | Round | Convex | 2.5×3 |
| $Y_2$ | Cream | Smooth | Entire | Round | Convex | 2.5×2.5 |
| $Y_3$ | Cream | Smooth | Entire | Round | Convex | 2.5×2 |
| $Y_4$ | Cream | Smooth | Entire | Round | Convex | 3×3 |
| $Y_5$ | Cream | Smooth | Entire | Round | Convex | 3×3 |
| $Y_6$ | Cream | Smooth | Entire | Round | Convex | 3×3 |

The macroscopic characteristics of isolated yeast from soil and different food samples were found to be cream or white colored, oval or round shaped, entire or lobated margin with a smooth texture (Thapa et al., 2015). Seven yeast cultures isolated from soil, fruit, and fermented products formed round, smooth, and cream-colored colonies ranging in size from 3.8 × 16.0 to 6.0 × 13.0 ( Ali & Khan, 2014).

Lactophenol Cotton Blue Staining was done to observe the shape of the yeast cell and the formation of buds under the microscope. Table 5-6.

**Table 5: Microscopic characteristics of Alcohol Tolerant Yeast Strains from Molasses Medium Based Cultures**

| Sample | Shape | Cell Arrangement | Buds |
|---|---|---|---|
| $C_M$ | Oval | Isolated or small clusters | Present |
| $M_1$ | Oval | Isolated | Present |
| $M_2$ | Oval and elongated | Isolated or small clusters | Present |
| $M_3$ | Oval and elongated | Isolated | Present |
| $M_4$ | Oval and elongated | Isolated | Present |
| $M_5$ | Oval and elongated | Isolated or small clusters | Present |
| $M_6$ | Oval and elongated | Isolated or small clusters | Present |

**Table 6: Microscopic characteristics of Alcohol Tolerant Yeast Strains from YPD Broth Medium Based Cultures**

| Sample | Shape | Cell Arrangement | Buds |
|---|---|---|---|
| $C_Y$ | Oval and round | Isolated or small clusters | Present |
| $Y_1$ | Oval and round | Isolated | Present |
| $Y_2$ | Oval and round | Isolated | Present |
| $Y_3$ | Oval and round | Isolated | Present |
| $Y_4$ | Oval and round | Isolated or small clusters | Present |
| $Y_5$ | Oval and round | Isolated or small clusters | Present |
| $Y_6$ | Oval and round | Isolated or small clusters | Present |

The cell shape of all the alcohol-tolerant yeast strains from molasses medium-based cultures was found to be oval, and as the concentration of alcohol increased, elongated cells appeared along with the oval cell. The cell arrangement was isolated, and some of them formed tiny clusters. Budding was observed in all cultures. In the case of all the alcohol-tolerant yeast strains from YPD Broth medium-based cultures, the cell shape was oval and round. The cell arrangement was isolated, with some forming small clusters. Budding appeared in all the cultures.

Yeast cultures isolated from soil and different food samples appeared as round or oval cells that were dark purple in color, and budding was also visible under a microscope (Thapa et al., 2015). The cells of the seven isolated yeast cultures from soil, fruit, and fermented items were oval, elongated, ovoid to spherical when young, and hexagonal with age. Cells exhibited oval, globose, spherical, and ellipsoidal budding ( Ali & Khan, 2014).

A total of 15 yeast cultures isolated from sugar-rich sources, including Grapes, Molasses, Mosambi, Cashew apple, Sugarcane, Sorghum and Distillery effluents, were observed for *Saccharomyces* characteristic, oval cell shape and budding characteristics. Seven of the fifteen isolates had an oval cell shape and showed budding character, identifying them as Yeast Grape (YGP), Yeast Molasses (YMO), Yeast Mosambi (YMI), Yeast Cashew Apple (YCA), Yeast Sorghum (YSM), and Yeast Distillery effluent (YDE) (Tikka et al., 2013).

The Pellet Formation of alcohol-tolerant strains was examined by inoculating the yeast cultures in YPD Broth Medium followed by 48-hour incubation.

Pellicle formation was not observed in any of the cultures of alcohol-tolerant yeast strains isolated from molasses medium-based culture as well as YPD broth medium-based cultures. Isolated yeast cultures from a soil sample, fruit sample and fermented products were compared with the reference strain of *Saccharomyces cerevisiae* MTCC 170 and showed that no pellicle was formed by any of the isolated yeast cultures, including the reference strain (Ali & Khan, 2014).

The Carbohydrate Fermentation Test was carried out to determine the ability of the alcohol-tolerant yeast strains isolated to ferment specific carbohydrates by inoculating cultures of yeast in basal medium with Durham's tube and the indicator methyl red. The test was carried outusing nine different carbohydrate sources which were numbered as Dextrose-1, Starch-2, Fructose-3, Sucrose-4, Galactose-5, Trehalose-6, α-Methylglucoside-7, Mannitol-8 and Maltose-9. The results are shown in Table 7.

**Table 7: Carbohydrate Fermentation Test of Alcohol Tolerant Yeast Strains from Molasses Medium-Based Cultures and YPD Broth Medium-Based Cultures**

| Type of Sugar | Sample | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | $C_M$ | $C_Y$ | $M_1$ | $Y_1$ | $M_2$ | $Y_2$ | $M_3$ | $Y_3$ | $M_4$ | $Y_4$ | $M_5$ | $Y_5$ | $M_6$ | $Y_6$ |
| Dextrose | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Starch | - | - | - | - | - | - | - | - | - | - | - | - | - | - |
| Fructose | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Sucrose | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Galactose | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Trehalose | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| αMethylglucoside | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Mannitol | + | + | + | + | + | + | + | + | + | + | + | + | + | + |
| Maltose | + | + | + | + | + | + | + | + | + | + | + | + | + | + |

(+) Positive, (-) Negative

All the alcohol-tolerant strains isolated from molasses-based cultures and YPD broth-based cultures were observed to ferment reducing sugars (Dextrose, Fructose, Galactose, Trehalose, Mannitol, and Maltose) and non-reducing sugars (Sucrose and α-Methylglucoside)after 72 Hours of incubation. However, the strains isolated were unable to ferment the polysaccharide starch.

The isolated yeast strains S1, S2 and S3 from sugarcane molasses, dates and figs were able to ferment sugar like glucose, galactose, sucrose, maltose and fructose, and then the strains were reported as *Saccharomyces cerevisiae* (Kechkar et al., 2019). The carbohydrate fermentation ability of the yeasts isolated from five species, guava, grapefruit, avocado, papaya, and gishitashowed, varied in utilizationthe of eight different sugars. Almost all isolates utilized glucose, galactose, sucrose, maltose, fructose and trehalose. All isolates failed to grow on xylose and lactose (Taye Negera, 2017).

Toddy was used for the isolation of yeast, and the results showed that the isolate was able to ferment sugars like glucose, galactose, maltose, sucrose, raffinose, and cellulose, which was identified as *Saccharomyces cerevisiae* (Kumar et al., 2011). According to the morphological and biochemical characteristics, all the isolated alcohol-tolerant strains are confirmed to be *Saccharomyces cerevisiae.*

**Fermentation Efficiency of Alcohol-Tolerant Strains**

The Yeast Cultures were kept for anaerobic fermentation followed by distillation and calculated for the fermentation efficiency of isolated alcohol-tolerant strains.

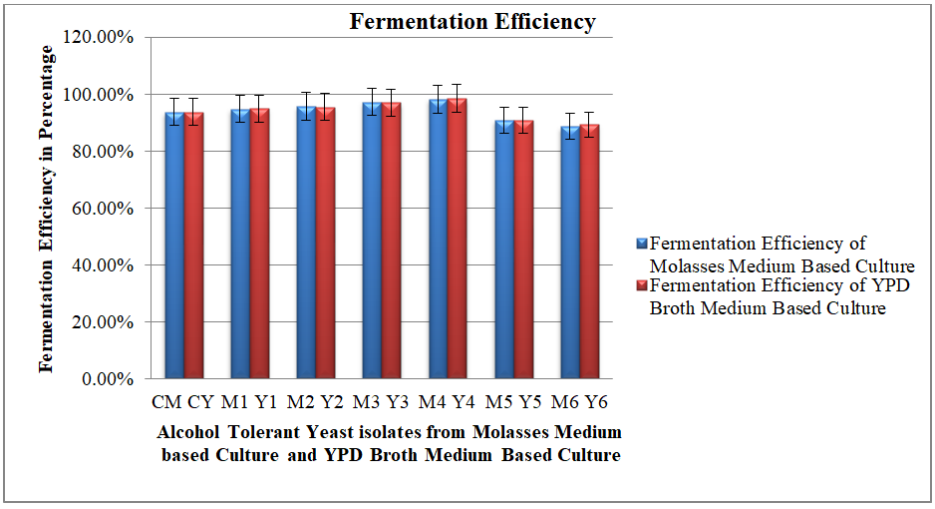


**Figure 7: Fermentation Efficiency of Alcohol Tolerant yeast isolates from Molasses and YPD Broth Medium Based Cultures**

The fermentation efficiency of control ($C_M$ and $C_Y$) was found to be 93.7%. The isolated alcohol-tolerant yeast strains, $M_4$ and $Y_4$, having an alcohol strength of 16%, gave the highest fermentation efficiency, which is 98.2% and 98.3%, respectively, which was higher even than the control. The alcohol-tolerant strains of 18% and 20% were found to have less fermentation efficiency in the 88-91% range. The thirteen morphologically different yeast strains, YC 1-13, were isolated from the Cassava root tubers sample, out of which only three were able to grow in 10% (v/v) ethanol or above. Strain YC3, YC9 and YC10 showed tolerance towards 12%, 12% and 11% ethanol concentration respectively (Ekunsanmi et al., 1990). Seven yeast cultures isolated from soil, fruit, and fermented products were compared to the reference strain *Saccharomyces cerevisiae* MTCC 170 and it found that *S. cerevisiae* C2 and TA strains had the highest ethanol tolerance (14% concentration) compared to other strains. The other strains may tolerate up to 12% ethanol content (Ali & Khan, 2014). The 15 yeast cultures isolated from sugar-rich sources, including grapes, molasses, mosambi, cashew apple, sugarcane, sorghum, and distillery, showed ethanol tolerance levels ranging from 7% to 12%. Although some strains showed a tolerance of 13%, growth was lower. Yeast Distillery effluent (YDE) has the maximum tolerance, up to 12%, compared to other strains (Tikka et al., 2013).

## CONCLUSION

In the present study, twelvealcohol-tolerant strains were developed from Molasses Medium based cultures ($M_1$, $M_2$, $M_3$, $M_4$, $M_5$ and $M_6$) and YPD Broth Medium based cultures ($Y_1$, $Y_2$, $Y_3$, $Y_4$, $Y_5$, and $Y_6$). The experimental results showed that all the developed alcohol tolerant strains could grow successfully at 10%, 12%, 14%, 16%, 18% and 20% concentration of alcohol. The developed alcohol-tolerant strains were characterized by conventional morphological and biochemical methods by comparing them with the wild commercial yeast cultures. The tests performed confirmed that all the developed alcohol-tolerant strains of *Saccharomyces cerevisiae*. The efficiency of developed alcohol-tolerant yeast strains in producing ethanol was evaluated for all the isolates. All the developed alcohol-tolerant strains were able to produce ethanol and showed tolerant characteristics for different alcohol concentrations. The fermentation efficiency of control yeast ($C_M$ and $C_Y$) was found to be 93.7%. Alcohol-tolerant strains of 18% and 20% alcohol strength showed fermentation efficiency in the range of 88%-91%. The alcohol-tolerant strains viz$M_4$ and $Y_4$ with 16% alcohol strength had the maximum fermentation efficiency of 98.2% and 98.3%, respectively. The alcohol-tolerant yeast strains - $M_4$ and $Y_4$ could serve as potential strains for fermentation even under high ethanol concentration and could be used at the industrial level for the fermentation of various raw materials in order to obtain an increased production of bioethanol.

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Ananthalakshmi Ranganathan, Anjali Yadav, and Shubhi Bhagwat were involved in the research. Alka Gupta and Shivam Pandey helped with the analysis, and Seema Paraha and Sonali Nigam reviewed the paper.

**Conflict of Interest**

The author declares that they have no conflict of interest.

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