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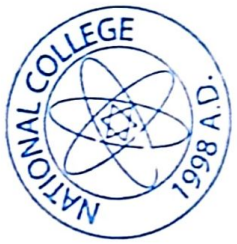

DETERMINATION OF ETHANOL TOLERANCE IN YEAST CULTURES ISOLATED FROM INDIGENOUS STARTER *MURCHA* AND THE EFFECTS OF UV MUTATION ON ETHANOL TOLERANCE

A Dissertation Submitted to **Department of Microbiology, National College**, Lainchaur, Kathmandu, Nepal, in Partial Fulfillment of the Requirements for the Award of Degree of Master's in Science in Microbiology **(Food and Industrial)**

By:

Ashish Nepal

TU Regd No. 5-2-0366-0032-2014

Symbol No: MB 1409/075

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DECLARATION

This dissertation entitled "**Determination of ethanol tolerance in yeast cultures isolated from indigenous starter *murcha* and the effects of UV mutation on ethanol tolerance**" has been submitted to the Department of Microbiology, National College, Tribhuvan University (T.U.), for the partial fulfillment of the requirements to the degree of Master of Science in Microbiology. This dissertation is conducted under the supervision of **Dr. Tika Bahadur Karki** and **Mr. Ashik Tiwari.** This is an original report of my research, has been conducted entirely by myself, and has not been submitted for any other degree or professional qualification. I have followed Tribhuvan University's current research ethics guidelines and accept responsibility for the conduct of the procedure in accordance with the University's rules and regulations.

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RECOMMENDATION

This is to certify that Mr. Ashish Nepal has completed the dissertation entitled "**Determination of ethanol tolerance in yeast cultures isolated from indigenous starter *murcha* and the effects of UV mutation on ethanol tolerance**" as a partial fulfillment of the requirements of Tribhuvan University for the completion of Master's Degree in Microbiology (Food) under our supervision. To our knowledge, this work has not been submitted for any other degree.

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On the recommendation of **Prof. Dr. Tika Bahadur Karki** and **Mr. Ashik Tiwari,** this dissertation by **Mr. Ashish Nepal** entitled "**determination of ethanol tolerance in yeast cultures isolated from indigenous starter *murcha* and the effects of UV mutation on ethanol tolerance**" has been approved for the examination and is submitted to the Tribhuvan University in partial fulfillment of the requirement of M.Sc. Degree in Microbiology (Food and Industrial).

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Title of the dissertation: **Determination of ethanol tolerance in yeast cultures isolated from indigenous starter *murcha* and the effects of UV mutation on ethanol tolerance**

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Supervisors: Prof. Dr. Tika Bahadur Karki and Mr. Ashik Tiwari

ABSTRACT

Microorganisms improved through various methods of strain improvement have been used in industrial production of various organic compounds efficiently for consumption or use in food production. The use of yeast in food grade alcohol production from sugar may be the most known of these processes. In recent years, the research has shifted from the optimization of the reaction parameters to the improvement of the microorganism used. Strain improvement, usually done by gene manipulation are more precise but also tend to be cost-prohibitive. A simpler method to bring about improvement is by mutation of the microorganism. The primary objective of the investigation was the isolation and identification of fermentative yeast from an indigenous starter culture followed by improvement of the alcohol tolerance through UV mutation. The study took place in the laboratory of National College, Khusibu from March 2022 to July 2022. In the present study, 21 isolates were obtained from murcha samples of which 8 were found to be fermentative yeasts. These isolates were subjected to identification, and tolerance tests to sugar and alcohol followed by mutation of best-performing isolates. Isolates Y_{10} and Y_{11} were identified as having the best tolerance to sugar and alcohol and were subsequently exposed to UV radiation for mutation. The growth as observed through UV spectrophotometry at 600nm increased for isolate Y_{10} from 0.89 to 1.55, 0.54 to 0.97, 0.23 to 0.43 at 0%, 5%, 10% respectively, and an increase from 0.73 to 1.1.59, 0.57 to 0.89, 0.23 to 0.6 at 0%, 5%, 10% respectively for isolate Y_{11}. Isolates Y_{10} and Y_{11} also grew at 12.5% of ethanol after mutation but they were not able to before UV treatment. The isolates Y10 and Y11 were identified as *Saccharomyces* spp. The mutated isolates were found to have increased tolerance to ethanol in terms of ethanol

percentage while all mutated isolates were found to have more growth in the media than the unmutated isolates after UV irradiation.

Keywords: Yeast, Murcha, Indigenous Starter, Sugar Tolerance, Alcohol Tolerance, Mutation

सोधसार

स्ट्रेन सुधारका विभिन्न विधिहरू मार्फत सुधारिएका सूक्ष्मजीवहरू विभिन्न जैविक यौगिकहरूको औद्योगिक उत्पादनमा प्रभावकारी रूपमा उपभोग वा खाद्य उत्पादनमा प्रयोग गर्न प्रयोग गरिन्छ। चिनीबाट फूड ग्रेड अल्कोहल उत्पादनमा खमीरको प्रयोग यी प्रक्रियाहरू मध्ये सबैभन्दा ज्ञात हुन सक्छ। हालका वर्षहरूमा, अनुसन्धान प्रतिक्रिया मापदण्डहरूको अनुकूलनबाट प्रयोग गरिएको सूक्ष्मजीवको सुधारमा सरेको छ। तनाव सुधार, सामान्यतया जीन हेरफेर द्वारा गरिन्छ अधिक सटीक तर लागत-प्रतिषेधात्मक पनि हुन्छ। सुधार ल्याउने सरल तरिका भनेको सूक्ष्मजीवको उत्परिवर्तन हो। अनुसन्धानको प्राथमिक उद्देश्य यूवी उत्परिवर्तन मार्फत अल्कोहल सहिष्णुतामा सुधार पछि स्वदेशी स्टार्टर संस्कृतिबाट किण्वन खमीरको अलगाव र पहिचान थियो। यो अध्ययन नेशनल कलेज खुसिबुको प्रयोगशालामा मार्च २०२२ देखि जुलाई २०२२ सम्म गरिएको थियो। हालको अध्ययनमा मुर्चा नमूनाहरूबाट २१ वटा आइसोलेटहरू प्राप्त गरिएको थियो जसमध्ये ८ वटा किण्वनयुक्त खमीर भएको पाइयो। यी स्ट्रेनहरू पहिचान, र चिनी र अल्कोहलको सहिष्णुता परीक्षणको अधीनमा थिए र त्यसपछि उत्कृष्ट प्रदर्शन गर्ने स्ट्रेनहरूको उत्परिवर्तन। आइसोलेट्स Y10 र Y11 लाई चिनी र रक्सीको लागि उत्तम सहिष्णुता भएको पहिचान गरियो र पछि उत्परिवर्तनको लागि यूवी विकिरणको सम्पर्कमा आए। 600nm मा UV स्पेक्ट्रोफोटोमेट्री मार्फत अवलोकन गरिएको वृद्धि पृथक Y10 को लागि 0.89 बाट 1.55, 0.54 देखि 0.97, 0.23 देखि 0.43 मा क्रमशः 0%, 5%, 10%, र 0.73 बाट 1.89, 1.70,1.59 मा वृद्धि भएको छ। पृथक Y11 को लागि 0.23 देखि 0.6 क्रमशः 0%, 5%, 10%। पृथक Y10 र Y11 पनि उत्परिवर्तन पछि इथेनॉल को 12.5% मा बढ्यो तर तिनीहरू UV उपचार अघि सक्षम थिएनन्। आइसोलेट्स Y10 र Y11 लाई *Saccharomyces* spp. को रूपमा पहिचान गरिएको थियो। उत्परिवर्तित पृथकहरूले इथेनोल प्रतिशतको हिसाबले इथेनोलमा सहिष्णुता बढाएको पाइयो जबकि सबै उत्परिवर्तित आइसोलेट्सले यूवी विकिरण पछि अनम्युटेड आइसोलेट्स भन्दा मिडियामा बढी वृद्धि भएको पाइयो।

कीवर्ड: खमीर, मुर्चा, स्वदेशी स्टार्टर, चिनी सहिष्णुता, अल्कोहल सहिष्णुता, उत्परिवर्तन

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ABBREVIATIONS

UV – Ultraviolet

DNA - Deoxyribonucleic Acid

OD – Optical Density

CFU – colony forming units

YMA – Yeast Malt Agar

YMB – Yeast Malt Broth

g - grams

ml - milliliter

^{o}C – degree Celsius

Chapter I

INTRODUCTION

1.1 Background

Ethanol is one of the most promising alternative fuel sources to traditional petroleum fuel available in the market (Capodaglio and Bolognesi, 2019). Ethanol, also known as ethyl alcohol, is a chemical compound consisting of a hydroxyl group attached to an ethane molecule. This chemical structure allows it to be used as a renewable fuel source, which can help reduce greenhouse gas emissions and dependency on fossil fuels. The potential for ethanol to be blended with gasoline in various proportions makes it a versatile option for powering vehicles, and its use has been steadily increasing in many countries due to supportive government policies and incentives (de Oliveira Gonçalves, 2023).

Ethanol is produced through both chemical and biological means. Most industrial-use ethanol is produced chemically, utilizing processes such as the hydration of ethylene. However, the ethanol produced for consumption is generally obtained biologically through the fermentation of carbohydrates. This fermentation process involves the conversion of sugars from agricultural crops like corn, sugarcane, rice, and other grains into alcohol (Coelho et al., 2008). This method not only provides a use for surplus agricultural products but also promotes sustainable farming practices. Furthermore, advances in biotechnology are continuously improving the efficiency and sustainability of ethanol production, making it an increasingly viable alternative to traditional methods of ethanol production.

Ethanol is one of the most used compounds in industrial and consumer products in terms of volume. The most common use is as a solvent or as an intermediate in the production of other chemical compounds, drugs, plastics, cosmetics, and lacquers. Ethanol is used in drugs as an antiseptic in topical medication and also as an antidote for methanol poisoning. Excluding ethanol used as and in beverages, they are also used in making perfumes, colognes, mouthwashes, and

aftershaves (Strohm, 2014). ethanol being easier and more effective than petroleum while also having a lower production cost. Ethanol is not just used as a pure replacement to fuel, it can be added or blended with gasoline to lower fuel costs at no to very little negative effects.

Fermentation is the biochemical anaerobic metabolism of sugars, a process that occurs in the absence of oxygen. This reaction is influenced by various factors, including temperature, pH, and the concentration of substrates and the product (Pelczar et al., 1993). Optimal conditions for fermentation vary depending on the type of organism and the specific substrates used. For instance, yeast, commonly used in ethanol production, has an optimal temperature range and pH level that must be maintained to maximize ethanol yield. Deviations from these optimal conditions can significantly impact the efficiency of the fermentation process, leading to reduced ethanol production and increased production costs.

It has been observed that most industrial fermentation processes for ethanol end prematurely, even when substrates are still available. Early studies attributed this premature cessation to a phenomenon known as the “stalling effect,” where the buildup of toxic end products inhibits further fermentation. Research by Ingram and Buttke (1984) identified ethanol itself as the primary end product responsible for this stalling effect. As ethanol concentrations increase, they become toxic to the fermenting organisms, hindering their metabolic activity and ultimately halting the fermentation process. Understanding this inhibitory effect of ethanol is crucial for improving industrial fermentation practices. Strategies such as the development of ethanol-tolerant microbial strains and the implementation of continuous removal systems for ethanol have been explored to mitigate this issue and enhance overall ethanol production efficiency.

Food-grade ethanol production on an industrial scale predominantly involves the use of microbial organisms, particularly yeasts. While certain bacteria can produce ethanol, yeasts are the most common organisms utilized for the fermentation of sugars into ethanol. Yeasts are eukaryotic, unicellular fungi naturally found in various environments, including soil. There are over 1,000

known species of yeast, though this number likely represents only a fraction of their true biodiversity (Kurtzman et al., 2011). The specific strains of yeast and their populations in any given environment are influenced by factors such as temperature, pH, and nutrient availability, which shape their growth and metabolic activities.

Yeasts have been harnessed for their fermentative properties for centuries, playing a crucial role in the production of alcoholic beverages and bread. Among the many yeast species, *Saccharomyces cerevisiae* is the most widely used in industrial ethanol production due to its high efficiency in fermenting sugars. The conditions under which these yeasts operate, such as optimal temperature and pH levels, are meticulously controlled to maximize ethanol yield. Additionally, the genetic and metabolic diversity among yeast species provides opportunities for selecting and engineering strains with enhanced fermentative capabilities and stress tolerance. Advances in biotechnology and genetic engineering have further improved the performance of yeast strains, enabling more efficient and sustainable production processes (Walker, 2003). As a result, the ethanol industry continues to evolve, driven by innovations that enhance yeast performance and process optimization.

Selection of yeast strains for fermentation is primarily based on their fermentation efficacy, which includes their ability to efficiently convert sugars into ethanol. Critical factors such as temperature and pH tolerance significantly impact the production process, as strains with narrow tolerance ranges require more precise and costly environmental controls. Additionally, yeast strains must be selected for their tolerance to high sugar concentrations, which serve as the substrate, and to ethanol, the end product. Strains that can withstand higher ethanol concentrations are particularly valuable because they can circumvent the stalling effect that typically halts fermentation when ethanol levels become toxic to the organisms. This tolerance is crucial for maintaining continuous and efficient ethanol production, ultimately improving overall yield (Gray, 1941). Moreover, advancements in genetic engineering and selective breeding have enabled the development of yeast strains with enhanced stress resistance and

metabolic efficiency, further optimizing the fermentation process and reducing production costs.

Yield improvement in ethanol production can be achieved through several methods, including optimization of the production process and enhancement of the yeast strains used. Process optimization involves fine-tuning various parameters such as temperature, pH, and pressure to create an ideal environment for fermentation. Maintaining optimal conditions ensures maximum yeast activity and efficiency in converting sugars into ethanol (Miah et al., 2022). This approach can significantly reduce production costs and increase yield by preventing the stalling effect and minimizing the loss of substrates. Additionally, continuous monitoring and adjustment of these parameters can help mitigate the effects of any fluctuations in the production environment, ensuring a stable and efficient process.

Improving yeast strains is another critical method for enhancing yield. In contemporary practices, strain improvement is often achieved through recombinant DNA technology and other gene improvement processes. These methods allow for precise modifications to the yeast genome, enabling the development of strains with enhanced fermentative capabilities, stress tolerance, and ethanol resistance. Mutation is another approach to strain improvement, which involves inducing genetic mutations to create beneficial traits. While mutations do not allow for precise genome adjustments, they can be achieved at a lower cost and with less technical complexity compared to recombinant techniques. Mutations can occur naturally or be induced using chemicals and/or radiation, providing a broad range of genetic diversity from which improved strains can be selected (Weber et al., 2017). Both genetic engineering and mutation breeding have proven to be valuable tools in developing yeast strains that enhance ethanol production efficiency and yield, contributing to the overall sustainability and profitability of the biofuel industry.

Despite its popular use and massive potential, industrial bioethanol production faces challenges. Notably, the toxicity of ethanol to the yeast *Saccharomyces cerevisiae* limits ethanol yield and productivity (Bai et al., 2004). The intricate

ethanol tolerance in *S. cerevisiae* results from a combination of genetic and environmental factors. Although extensive research has been conducted, no single genetic modification significantly enhances ethanol tolerance at high concentrations. Therefore, optimizing yeast strains through mutation and other genetic techniques remains crucial for advancing bioethanol production technologies (Lam et al., 2014).

Developing yeast strains with improved ethanol tolerance can revolutionize bioethanol production (Ali, 2014). These benefits include increased ethanol yield, enhanced fermentation kinetics, reduced inhibition, and positive economic and environmental impacts (Ünaldi Coral, 2002). Tolerant strains can thrive and produce ethanol at higher concentrations, leading to overall higher yields. Additionally, improved tolerance reduces lag phases and accelerates fermentation rates, ultimately shortening production times. Such advancements make bioethanol production more efficient. Improvement through easy means of mutation may be used in other organisms that are used in fermentation for production of various organic products.

1.2 Objectives

1.2.1 General Objective

- To screen ethanol tolerant yeast cultures from *Murcha* and improvement of these isolates by mutation

1.2.2 Specific Objectives

- To isolate and screen fermenting yeast isolates from indigenous starter *Murcha.*
- To determine tolerance of selected isolates to different ethanol concentrations.
- To induce mutation in the isolates by UV radiation
- To compare ethanol tolerance effects on isolates
- To identify yeast isolates

Chapter II

LITERATURE REVIEW

2.1 Ethanol

Ethanol or Ethyl alcohol (C_2H_5OH) is an organic compound that exists as a colourless, volatile, and liquid that burns with a blue flame without any smoke (NCBI, 2022). Ethanol is one of the more important organic compounds, not only alcohols, and it is available easily as a worldwide commodity. Ethanol has a characteristic odour and taste but is judged to be pleasant. Physical and chemical properties of ethanol are the effect of the hydroxyl (-OH) group.

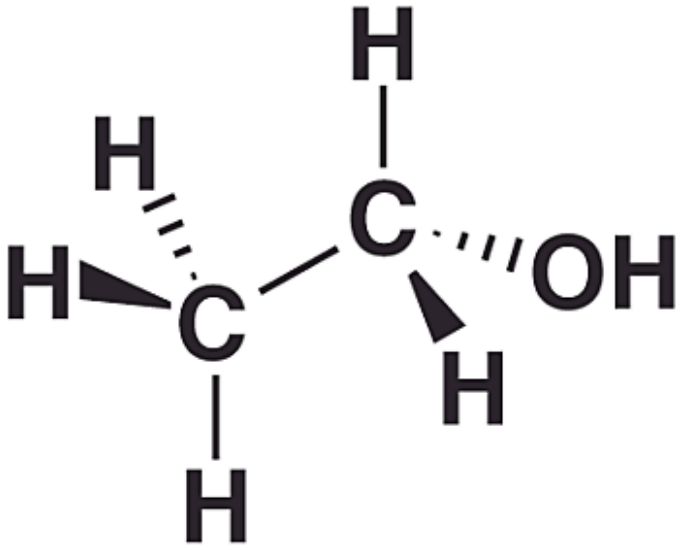


Fig 1: Ethanol Structure

Ethanol has numerous uses. It is used primarily as a solvent, and also as a germicide, antifreeze, beverage, biofuel, additive to fuels and paints, in cosmetics like perfumes, deodorants, beauty products, and lubricants (Criddle et al., 2018). Ethanol is also a major intermediate for industrial synthesis of other organic compounds, mainly acetic acid. Use of ethanol as biofuel is a rising (Bhatia, 2014). Vehicles that utilize purely alcohol or ethanol added fuel are environment friendly alternative to traditional fuel-powered vehicles. Its use

as a beverage is also a big contributor to ethanol being the most utilized organic compound in the world. Ethanol is a central nervous system depressant resulting in effects of sedation and hypnosis. In small doses, this effect is thought to be stimulation as inhibitory systems are suppressed. In large doses, however ethanol may cause coma and death among other effects of its toxicity (Clarke, 2007).

2.2 Ethanol Production

Ethanol is one of the most produced compounds industrially; 10.322 trillion litres in 2021 (Renewable Fuels Association, 2022). Production may be using chemicals or by fermentation. Chemical process may be direct or indirect hydration of ethylene. The direct process was introduced by Shell in 1947 and involved the catalytic direct hydration of ethylene (Weissermel and Arpe, 2003). The process is reversible and exothermic, and the reaction happens between ethylene and water vapours. The process happens in three steps consisting of the reaction, the recovery, and purification resulting in the production of anhydrous ethanol. The equations involved are:

$$CH_2{=}CH_2 + H_2O \leftrightarrow CH_3CH_2OH$$

$$C_2H_2 + H_2O \leftrightarrow CH_3CHO$$

$$2C_2H_5OH \leftrightarrow H_2O + (CH_3CH_2)_2O$$

The process is 4-5% efficient under this condition and the ethylene is recycled (Hidzir et al., 2014). However, since ethylene is a petroleum compound, the downside of petroleum is being taken more seriously in the industrial sphere. As such, fermentation is the most common method of ethanol production, especially for consumption.

The other method involves the use of microorganisms and fermentation process. Alcohol production happens by catalysis of sugars or complex carbohydrates (cellulose, starch) by action of yeasts alone or with other microbes in the presence of water. In the present day, 90-95% of ethanol produced is through fermentation. Fermentative product ethanol is processed further by distillation and dehydration (Sarris and Papanikolaou, 2016). Fermentation is the most

popular process as reaction substrate can be carbohydrate-heavy waste from other industrial processes.

2.3 Fermentation

Fermentation is a metabolic process that causes changes in organic compounds as a result of enzymes acting on it. In food production, this process may refer to a process where microorganisms bring about a selective change to a food or beverage. Fermentation is not a recent process and has been in use since ancient times. The first instance of fermentation dates back to around 7000 BC (Farnworth, 2008) when bread and cheese making were practiced. As such, almost every culture in the world has some form of fermented food, beverage or the use of fermentation as a preservation method in practice.

Fermentation is currently a very popular process in industrial production of various foods and beverages. These include but are not limited to bread, cheese, yoghurt, kimchi, sauerkraut, pickles, and beverages such as beer, cider, wine, spirits, kombucha, and hard seltzers. Organic compounds and enzymes like citric acid, acetic acid, xanthan gum, dextran, diacetyl, and glucose isomerase are also produced by fermentation. Fermentation also allows for large-scale production of some antibiotics that are produced by microorganisms.

The fermentation process can follow a few different methods before the actual fermentation process. The method is dependent on the type of substrate used. The substrates are classified on the basis of carbohydrate complexity (Onuki et al., 2016):

- Sucrose-containing substrate e.g.: Sugar beet, sweet sorghum, Sugar cane
- Starchy materials e.g.: wheat, corn, barley
- Lignocellulosic biomass e.g.: wood chips, grasses

The method of fermentation differs with the substrate used.

Starchy materials like corn and barley are milled first. The milling may be dry or wet. Dry milling involves grinding the entire kernel of grain into flour,

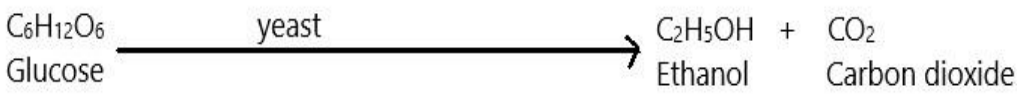
forming a mash, and then converting the starch into sugar by use of enzymes. This mash is then carried out to fermentation (Onuki et al., 2016). The process is cheap compared to other processes and also is a simpler process with fewer steps. Used grain can be used as animal feed (Rasmussen et al., 2014). It also produces less ethanol when compared to wet milling.

Wet milling involves steeping of the grains in water and Sulphur dioxide and separation of the kernel into its components like germ, fiber, gluten, and starch for 24-48 hours and using only the carbohydrate part for the fermentation. By products obtained during wet milling are useful in production of corn oil (from the germ), animal feed (from gluten), syrup and other sweeteners (from starch). (Renewable Fuels Association, 2015)The process produces more ethanol than dry milling, but the process is more complex and also requires higher upfront and operational costs than dry milling (Rausch et al., 2019).

Sucrose containing substrates like sugarcane, beet, etc. are juiced and then the juice is subjected to the fermentation process (Cardona and Sánchez, 2007).

Lignocellulosic biomass like agricultural residues, grass, and wood involves breaking down cellulose and lignin by enzymatic processes into fermentable sugars, followed by fermentation. These processes are listed in line with the capital required for the method (Hamelinck et al., 2005).

Alcohol production by fermentation is carried out in the presence of water by yeasts. While many yeasts can carry out fermentation, the yeasts from the genera *Saccharomyces* are mostly used. The reaction for the process can be visualized as (Criddle et al., 2018):



This reaction is influenced by temperature, pH, efficacy of microorganism used and the concentration of substrate and product in the fermenter for the reaction time (Pelczar et al., 1993).

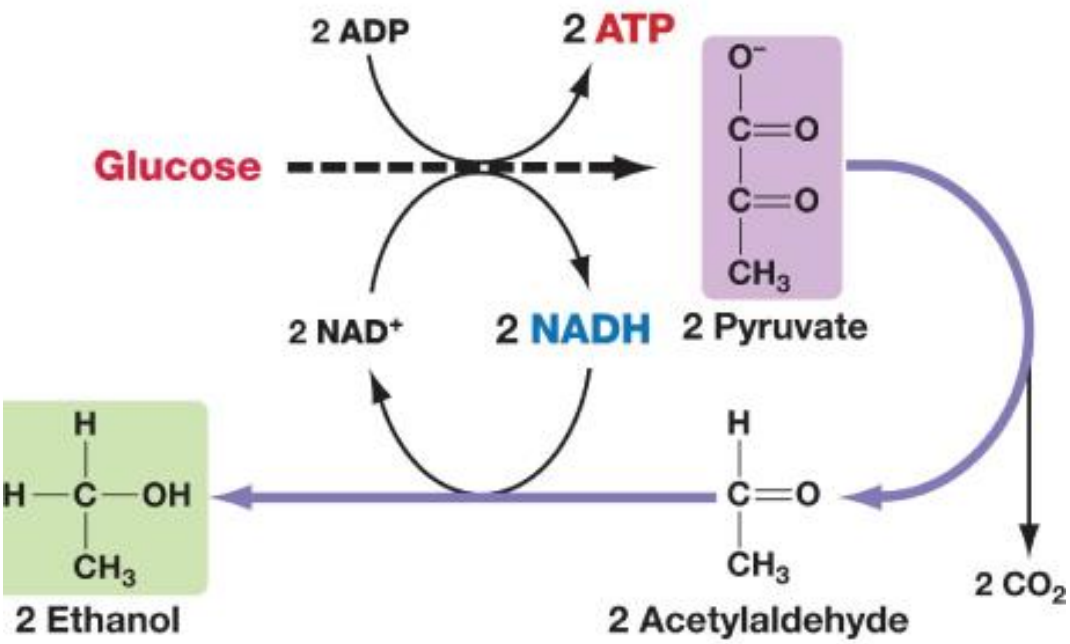


Fig 2: Alcohol Fermentation Pathway

Ethanol fermentation is mainly of the following types (Ishizaki and Hasumi, 2014):

- Batch Fermentation

  In this type of fermentation, the substrate and the fermenting microorganisms are added in fixed amounts and allowed to ferment until the substrate runs out. The products are then harvested. This method is used in breweries and wineries.

- Continuous Fermentation

  In this type of fermentation, substrate is added to the fermenter continuously with the removal of only the product continuously. This allows for a never-ending fermentation process and is useful in production of industrial ethanol due in part to its efficiency.

- Fed-batch Fermentation

  Fed-batch fermentation is a combination of the two methods above. In this method, the substrate is added into the fermenter in increments

without removal of the main culture fluid. This aids in controlling substrate concentration helping reaction efficiency and product yield.

2.4 Stalling Effect

Stalling effect in fermentation happens when the yeasts fail to catalyze the sugars in the fermentation medium as a result of imbalance in the reaction parameters. Yeasts maybe stalled as a result of imbalance reactant and product compounds, sugars and ethanol. Production of ethanol is limited by inhibition of fermenting organisms by the effect of ethanol produced affecting the osmotic balance in the reaction medium (Jiménez and Benítez, 1986). A very similar effect can be seen in case of sugar concentrations. The ethanol production increases up to a certain percentage of glucose in the substrate after which the yeasts get essentially deactivated and the reaction stalls. Statistical correlation test showed a strong positive correlation between glucose concentration and fermentation rate in an investigation (Bryan et al., 2018).

2.5 Murcha

Murcha is a traditional starter used by the people of central and eastern part of the Himalayas. Murcha is the Nepali word for the starter used in the region with other names used by other ethnic groups such as phab (Tibetans and Bhutia), khesung (Limbu), bharama (Tamang), bopkha or khabed (Rai), buth or thanbum (Lepcha), and poo (Drukpa). The preparation and trade of murcha is regarded as a closely guarded secret in these communities and are passed down generationally. Traditional murcha preparation is done at night to keep this art a secret (Tamang, 2009). Murcha in Nepal is found as two types: mana and manapu.

- Mana

  Mana is a granular type of mixed culture prepared from wheat flakes (Nikkuni et al., 1996). Preaparation begins with soaking of wheat grains overnight and then cooking. The grains are spread on the floor to cool and powdered murcha is added around the end of the cooling. The grains

are covered with paddy and fermented at 25^{o}C for 9-10 days until green mold appears. This mass is sundried and ready for use.

- Manapu

  Manapu is a mixed starter in the shape of cakes and prepared from rice and millet flour. The flour is mixed and kneaded with powdered murcha and shaped into cakes. The cakes are dried on paddy straw and left to ferment for 5 to 7 days at 25^{o}C. The fermented cakes are sundried and used. Indigenous preparation includes *manashawa* and black pepper (Tamang, 2009).

This starter culture is a mixed culture cake that consists of numerous saccharifying molds, fermentative yeast, and acidifying lactic acid bacteria with respective loads of 10^6 CFU/g, 10^8 CFU/g, and 10^7 CFU/g (Saono et al., 1986; Tamang and Sarkar, 1995). Although many species of microorganisms are present in murcha, the most desirable organisms include *S. cerevisiae*, *S. fibuligera*, *C. versatilis*, *Rhizopus* spp., and *P. pentosaceus* (Subba, 2012).

2.6 Yeast

Yeasts are microscopic fungi that are ubiquitous in nature. Yeasts are very important in the fermentation process. They convert sugar into alcohol, carbon dioxide and other chemical compounds. Yeasts used in ethanol fermentation are eukaryotic, unicellular and are mostly comprised of species from the *Saccharomyces* genus. They are usually oval or spherical and about 10µm in diameter and occur mostly in chains, clusters or pairs (Boulton and Quain, 2006). Based purely on technological ease, yeasts are classified into two groups: *Saccharomyces* and non-*Saccharomyces* (Walker and Stewart, 2016).

- Non-*Saccharomyces* Yeasts

  Non-*Saccharomyces* yeasts are a group of microorganisms useful in various fermentation processes as a function of their metabolic differences. While these yeasts are useful in fermentation, they were also considered as contaminants as they are able to modify the sensory

quality of wines in wineries. This is why these yeasts were eliminated or kept at a decreased level as a basic process in wine making (Ciani and Maccarelli, 1998). This perception has changed in recent years as research suggests that the action of these yeasts is very relevant in producing the positive sensory qualities of wine. In fermentation, these yeasts are the majority during the start of the process of spontaneous fermentation until the ethanol concentration reaches 4-5%, at which point, their growth slows as the dissolved oxygen is exhausted and ethanol concentration further rises. *Saccharomyces* then takes over and completes the fermentation. Some non-*Saccharomyces* persist until the end and effect the fermentation as a result of their metabolic processes. The positive sensory effects of these yeasts have therefore given rise to an increased interest in the nature and fermentative characteristics of non-*Saccharomyces* yeast (Estela-Escalante et al., 2017). Non-Saccharomyces yeasts with their lesser fermentative acumen and ethanol production, may be considered disadvantageous in fermentation but they also contribute to the production of esters, fatty acids and higher alcohol, which produce flavour in ethanol and this production of aromatic compounds is not possible through only Saccharomyces yeast (Cordero-Bueso et al., 2013). Use of a mixed culture of Saccharomyces and non-Saccharomyces yeast is therefore used in production of wider sensory characteristics needed to improve wines and beers (Canonico et al., 2016). Non-Saccharomyces yeasts that have been on the forefront of this research include Pichia, Candida, Kloeckera, Kluyveromyces, and Brettanomyces (Maicas, 2020).

They can be found on surface of fruits and vegetables, in plant exudates like sap, and also in the soil. Yeasts are also a major part of normal microbiological flora of various mammals and insects. Research suggests soil population of yeasts of 10^5-10^6 colony forming units per gram of soil suggest healthy growing cells (Phaff and Starmer, 1987). Besides the common yeast species like *Saccharomyces, Pichia*, etc., species like some *Cryptococcus, Debaromyces, Lipomyces* are exclusive to soil environments. In spite of this, yeast species are

mostly collected from plant and other organic matter. Di Menna 1957, reported 10^5-10^7 yeast cells per gram of fresh foliage. Plant matter including flowers and decaying fruits support populations as much as 10^6 cells/g (Phaff and Starmer, 1987).

2.7 Yeast Reproduction

Yeast Sporulation and budding are the main reproductive systems present in yeast cells. Sporulation is the sexual reproductive process and budding is the vegetative process. Sporulation occurs in yeast cells that are under adverse environmental conditions (Freese et al., 1982). Wine strains of *Saccharomyces cerevisiae* rarely express sporulation while they do possess the capability to sexually reproduce. Sporulation occurs during nutrient starvation, or addition of chemicals like sodium acetate or both in breeding experiments. The sporulation cycle in yeasts enables them to undergo hybridization, mutation, and recombination leading to evolutionary differences, which is why sporulation not desirable during fermentation (Neiman, 2005).

Vegetative reproduction occurs most commonly through budding in yeasts. This process preserves the characteristics of yeast cells across multiple generations which is desirable in fermentation. Buds appear at the ends of the ovoid structure of yeast cells, which is the area with the most curvature in the cell (Merlini et al., 2013). Buds may appear singly or in multiples on the same cell, never at the same place. Budding is complete when the bud develops on the mother cell and separates into its own sharing the same genome as the mother cell. Budding may give the appearance of short chains of cells during microscopy. Budding is desirable during cell growth before inoculation and at the early stages of fermentation (Jackson, 2020).

2.8 Yeast Fermentation Processes

Most microorganisms have the ability to ferment sugars, and they do so in the absence of oxygen in the growth environment. Fermentation is an inefficient mode of metabolic release, converting only 6-8% of the chemical bonds in glucose into metabolic energy (Jackson, 2020). Yeasts like Saccharomyces

however are unusual in that they are adapted to fermentative metabolism producing equivalent metabolic energy per second as normally garnered through reproduction (Pfeiffer et al., 2001). This adaptation is in part based on the presence of a high efficiency alcohol dehydrogenase enzyme and in part due to a mitochondrion that only produces enzymes used in respiration when the cell is in presence of non-fermentable substrate (Ihmels et al., 2005). *Saccharomyces* can also withstand somewhat high ethanol concentrations in addition to being osmotolerant, acid-tolerant, and able to thrive in low oxygen concentrations (Sree et al., 2000). Yeasts are used locally and industrially in the production of various food and non-food products. Some of these processes are:

2.8.1 Alcoholic Fermentation

Use of yeasts in production of alcoholic beverages is one of the oldest biotechnological processes. Yeasts play an important role in production of all alcoholic beverages and the yeasts selected have major effect on the alcohol yield and beverage sensory quality (Walker and Stewart, 2016). In wine fermentation, yeasts that can produce a higher ethanol yield are necessary. While *Saccharomyces* are the main yeast used and traditionally, non-*Saccharomyces* yeasts were considered contaminants, pasteurization is utilized as the method to rid of these yeasts in addition to adding sulphites (Maicas, 2020). In beer and cider fermentation, ethanol production can be less, but the unique flavours and aroma of the beverages must be preserved (Dzialo et al., 2017; Lorenzini et al., 2019). While wine, beer, and cider are the more popular and widely known beverages, yeasts are used locally in fermentation of locally available fruits and grains all over the world, like dates in North Africa, pineapples in Latin America, jackfruit in Asia, and banana beer in East Africa (Gensi et al., 2000).

2.8.2 Non-Alcoholic Fermentation

Yeasts are also necessary in food production processes other than alcohol. *Saccharomyces* sp better known as Baker's yeast, are the strain used in making bread. This fermentation is the most important phase in breadmaking. Yeasts produce CO_2 and other metabolic excesses that provide bread with its signature

volume, texture, and taste (Struyf et al., 2017). Yeasts used in bread production differ with the type of bread. In addition to bread, yeasts are useful in production of coffee (de Melo Pereira et al., 2014) and chocolate (Papalexandratou and Nielsen, 2016).

2.8.3 Biofuels and Other Chemicals

Yeasts are useful for the purpose of production of biofuels and other advanced chemicals in industries (Kwak et al., 2019).

2.9 Tolerance

It is known that fermentation, as any other chemical reaction is affected by parameters like pH, temperature, osmotic balance, and invertase activity. The ability of yeast strains to utilize sugars, tolerate high alcohol and sugar concentrations, produce ethanol, and determines the efficiency of the yeast strain in the industrial process (Matapathi et al., 2004). Very high concentrations of ethanol and non-optimal concentrations of sugars have been reported to stall fermentation. As such, tolerant yeast strains should be able to keep the reaction going on for longer and produce more ethanol during fermentation. These factors help in making the process more efficient.

Increased yield of ethanol by fermentation is dependent on the use of an ideal yeast strain, appropriate fermentation substrate, and proper process technology (Brooks, 2008). Additionally, tolerances to ethanol, carbon dioxide, glucose, are regarded as traits of a good yeast strain (Priest and Campbell, 2003).

2.10 Cell Density

Cell density, often measured as the number of cells per unit volume, is a critical parameter in yeast research and biotechnological applications. Accurate measurement of cell density is essential for optimizing fermentation processes, studying cell growth kinetics, and evaluating the effects of various treatments on yeast cultures. Optical density (OD) is a common proxy for cell density, especially in liquid cultures. By measuring the OD at a specific wavelength, typically 600 nm (OD600)(Beal et al., 2020), researchers can estimate the

concentration of yeast cells in suspension. The relationship between OD and cell density is established through calibration curves, where known concentrations of yeast cells are correlated with their respective OD readings. This technique is widely adopted due to its efficiency and reliability in providing quick estimates of yeast cell concentration, which is crucial for both experimental and industrial applications (Myers et al., 2013).

The spectrophotometer is a vital instrument in microbiology and biotechnology, enabling precise measurement of light absorbance across various wavelengths. This device is instrumental in quantifying the concentration of substances in solution, including microbial cultures. Spectrophotometers operate by passing light through a sample and measuring the intensity of light before and after it passes through (Myers et al., 2013). The difference in light intensity is used to determine the absorbance, which correlates with the concentration of the target analyte. In the context of yeast cell studies, spectrophotometry is particularly useful for assessing cell density by measuring the optical density (OD) of yeast cultures (Carvalho et al., 2021). This method is preferred due to its simplicity, speed, and non-destructive nature, allowing for continuous monitoring of yeast growth and metabolism.

2.11 Strain Improvement

Microorganisms generate a wide range of products in very low concentrations, which have been utilized as antibiotics, drugs, vitamins, enzymes, bulk organic compounds, polymers, amino acids, biofuels, and more (Acevedo-Rocha, 2018). For efficient biotechnological processes at an industrial scale, it is essential to use microbial strains that produce high levels of the desired product. However, this may not be a natural characteristic of the selected microorganisms; therefore, modifications in their genetic material could potentially help overcome this limitation. Consequently, industrially significant microbes undergo various treatments using physical, chemical, or genetic tools to overproduce the desired metabolite and make the process cost-effective. This enhancement of the biosynthetic capabilities of microbes to produce the desired

product in larger quantities is known as microbial strain improvement (Saxena, 2015).

The concept of microbial strain improvement includes the development of strains with an enhanced ability to utilize complex raw materials and efficiently assimilate them, making the process more economical, reduce or eliminate unwanted by-products of the microbial process, increase the extracellular release of the by-product, lower the toxic threshold of the end product to facilitate high accumulation with minimal cell death, shorten the fermentation period, and overproduce native or foreign products following genetic recombination (Adrio and Demain, 2010).

Yeast strains are generally resistant to ethanol up to a certain degree. However, some strains may be more tolerant of alcohol. This tolerance may further be increased and improved so that the fermentation does not stall even at high concentrations of ethanol (Jiménez and Benítez, 1986). The process of changing the inherent qualities of microbes for the improvement of the strain can be done by various methods. Methods using invitro gene manipulation techniques like recombinant DNA technology, hybridization, metabolic engineering, selection of high-yielding mutants, and mutagenesis (Ryu and Nam, 2000).

2.12 Mutation

Genes are segments of DNA molecules that are transmitted with great accuracy. Sometimes, these can vary a little during multiplication which brings about a change in the DNA and as a result, the organism (Cerutti, 1975). The changes that are inherited in the genome are known as mutations. Mutations are not necessarily bad; they have pros and cons. While there are mutations that can cause illnesses and disorders in cells which may cause death, some mutations are good. Mutations in plants and animals have improved tolerances to diseases and pests (Oladosu et al., 2016).

Mutation, depending on its origin, can be one of two types: spontaneous or induced. Spontaneous mutations occur naturally and are assumed to be random changes. The origin of spontaneous mutation is unknown. There are however

several mechanisms responsible for the phenomenon, namely, mis-pairing errors during replication, de-purination, deletions, insertion sequences, and error-prone DNA repair mechanism (Davisson et al., 2012).

Induced mutations are caused by artificial factors; physical and chemical agents, called mutagens. The simplicity of these techniques has massive appeal to researchers and these methods are considered classical mutagenesis. Physical agents include ionizing and non-ionizing radiation (X-rays, ɣ-rays, UV radiation) and chemical agents consist alkylating agents, intercalating agents, and base analogs (5-bromouracil, 5-chlorouracil, hydroxylamine (NH_2OH), nitrous acid (HNO_2), ethyl methane sulphonate, N-methyl-N'-nitro-N-nitrosoguanidine (Saxena, 2015).

2.13 Scientific Applications of Mutations (Past and Present)

M. Demerec was the first to use X-rays to isolate a mutant strain of Penicillium chrysogenum called X-1612, which produced three times more than the wild strain 1951-B25. Penicillium was also the first organism treated with UV rays, leading to the isolation of a mutant strain Q-176, which showed three times higher activity than the mutant X-1612 (Demerec, 1945). Scientists have used ultraviolet light (UV) to create improved versions of a fungus called Acremonium chrysogenum. This fungus is used to produce the antibiotic cephalosporin. One such improved strain, named M8650, was developed from the original fungus first isolated by Brotzu (Bo, 2000). Another successful strain, CW19, was created by Eli Lilly and Co. This strain produced three times more cephalosporin than the original one. By further optimizing the conditions in which the fungus grows (fermentation), scientists were able to boost cephalosporin C production by 15 times compared to the original fungus (Miller and Litsky, 1976).

To make penicillin production economically feasible, four research groups—the USDA Peoria laboratory, Carnegie Institute of Washington, University of Minnesota, University of Wisconsin, and Eli Lilly Industries—conducted mutagenesis using radiation and chemical mutagens (Elander, 2003). They employed nitrogen mustard (NM), ultraviolet radiation (UV), and X-radiation

(X), and also utilized spontaneous (S) mutation to improve the Penicillium chrysogenum NRRL-1951 strain. Through approximately 21 rounds of mutation and improvement, various laboratories increased penicillin yield by a factor of 55. Eli Lilly eventually developed a modified P. chrysogenum E15.1 strain, which produced 7 g/l of penicillin in shake flask conditions compared to the yield of P. chrysogenum NRRL-1951 as achieved by Florey (Elander, 1999). Further optimization of fermentation conditions in a bioreactor under submerged conditions increased the yield to 20 g/l of penicillin, marking the development of penicillin as the first commercial antibiotic (Kardos and Demain, 2011).

Site-directed mutagenesis, a technique that introduces specific mutations at chosen positions in a gene, is widely used in bioscience. Saturation mutagenesis, which introduces random mutations into a gene to create a set of all possible mutants, is also frequently employed in directed evolution to enhance or modify protein properties. Despite the development of various methods to introduce these mutations, there remains a demand for more efficient and convenient techniques. Recently, two promising methods emerged: ISO (one-step isothermal in vitro recombination) and its mutation-focused version, MISO (multi-change isothermal in vitro recombination). The ISO method uses three enzymes to join DNA fragments with short overlapping regions. While MISO has been shown to assemble multiple fragments and introduce up to eight mutations in a single gene, it wasn't originally designed for making precise changes at multiple locations within a gene (its focus was on building longer DNA pieces) (Saxena, 2015).

2.14 Mechanism of UV Mutation

UV radiation was discovered to be mutagenic in 1934. The basic action of UV light on DNA is the formation of pyrimidine dimers, usually thymine. The dimers disrupt the usual replication and causes changes in the resulting genome. The dimers form a cyclic compound by covalently bonding with adjacent pyrimidines, linking the two. This structure is not a regular DNA intermediate. This also blocks pyrimidine base pairing, resulting in a kink in the DNA

structure interrupting the process of translation, i.e. protein synthesis, and thereby halting replication (Cadet and Douki, 2018)

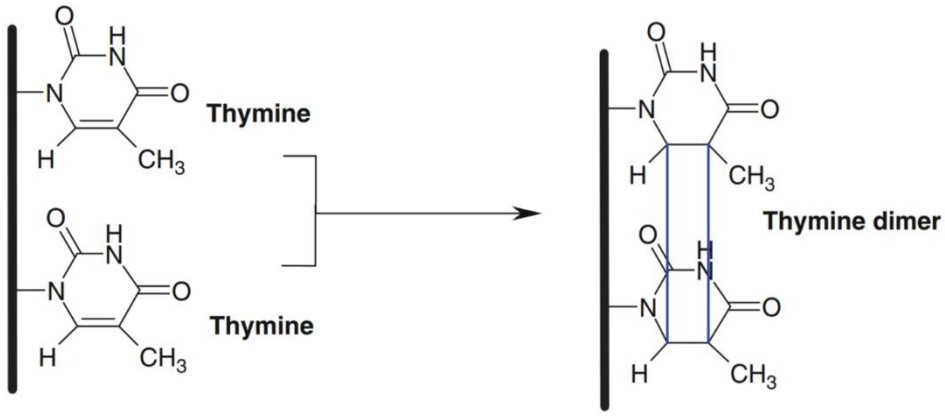


Figure 3: Thymine Dimer Formation

Additionally, Ultraviolet (UV) radiation can damage DNA by creating abnormal structures called 6-4 photoproducts. These photoproducts link neighboring pyrimidines (the building blocks of DNA) together at their 6th and 4th carbon atoms. Both these photoproducts and other UV-induced dimers disrupt accurate DNA copying, leading to errors when DNA is converted into instructions for building proteins (transcription) (Saxena, 2015).

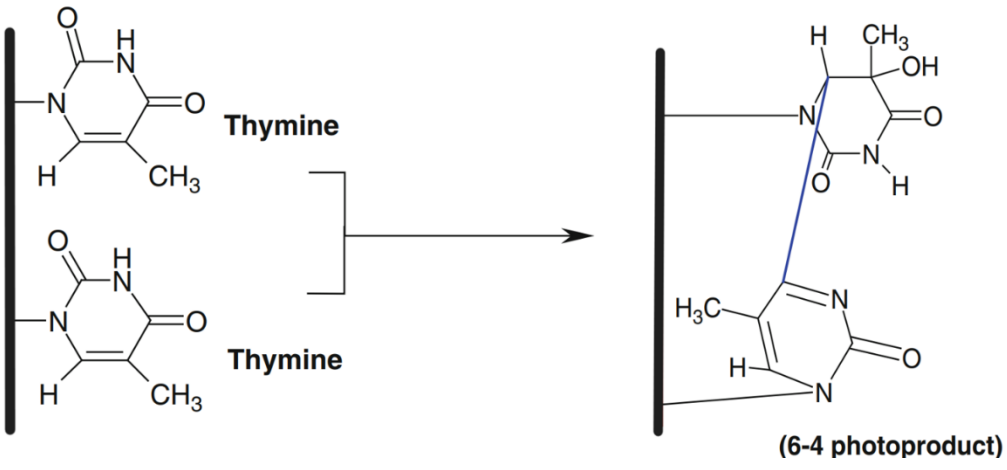


Figure 4: 6-4 photoproduct Formation

Chapter III

MATERIALS AND METHODS

3.1 Materials

The materials and equipment, glassware, chemicals, microbiological media, and other instruments required for this study are listed in appendix A.

3.2 Methods

3.2.1 Study Period

This study was conducted in the microbiological laboratory of National College, Khusibu, Kathmandu from March 2022 to July 2022.

3.2.2 Sample and sampling method

Murcha samples were collected from different parts of Nepal for isolation of yeast. Different samples of murcha (including manapu and mana) were collected for alcohol tolerant yeast. Altogether 21 manapu and 9 mana samples were collected for this research. A culture of *S.cerevisiae* from the wort formation phase was obtained from a local brewery.

3.2.3 Sample collection and transportation

Murcha samples were collected in sterile polythene plastic bags. Ten grams were taken and transported to the Food Microbiology Laboratory of National College. Collected samples were stored at room temperature until further processing.

S.cerevisiae obtained was transported to the laboratory in a media tube containing YMB. This was plated in YMA in the laboratory and stored after growth at 4^{o}C.

3.2.4 Sample processing

Five grams of murcha samples were grounded with the help of sterile mortar and pestle. The grinded murcha sample was mixed with 45 ml of sterile normal saline. Then it was thoroughly mixed to make the first dilution. Then, 1 ml of diluent was transferred to a tube with 9 ml sterile normal saline and referred as 10^{-2} dilution. The same process was to be repeated up to 10^{-6} dilution (Karki et al., 2017). After each dilution, the tube was shaken well to homogenize the sample. This process was repeated for all samples.

3.3 Isolation, Purification and Preservation of yeast cultures

3.3.1 Isolation

Diluents of the murcha samples were inoculated onto agar media plates. 0.1 ml of the dilutions from tubes 10^{-2}, 10^{-4}, 10^{-6} was pipetted out and spread onto YMA (Yeast Malt Agar) plates with the help of a bent glass rod. The media plates were incubated at 28^{o}C for 48 hours and the colonies growing on the agar surface were observed (Ünaldi Coral et al., 2002). Colonies that resembled yeast were selected on the basis of the growth characteristics on media surface.

3.3.2 Morphological Characteristics

All the isolates were aseptically transferred on the YMA plates and their morphological characteristics were observed and recorded based on colour, texture, margin, elevation, consistency, opacity etc (Laluce et al., 1993).

3.3.3 Screening of fermentative yeasts

Yeast colonies obtained were inoculated from the plates with an inoculating loop into test tubes with 5 ml Yeast Malt Broth and incubated for 24 hours at 28^{o}C. The media tubes were prepared with inverted Durham's tube. After incubation, the tubes were observed for turbidity, gas production, and alcoholic smell (Guimarães et al., 2006). The tubes that developed alcoholic smells and gas production indicating positive fermentation were sub-cultured on Yeast Malt media plates and incubated. After incubation, these colonies were further tested and studied.

YMB test tubes with inverted Durham's tube

Inoculation of a loopful of culture

Incubation at 28oC for 48 hrs

Observation of media tubes for turbidity, alcoholic smell, and gas formation in Durham's tube

Selection of potent yeast isolates (high alcoholic smell and gas formation with good growth in the media)

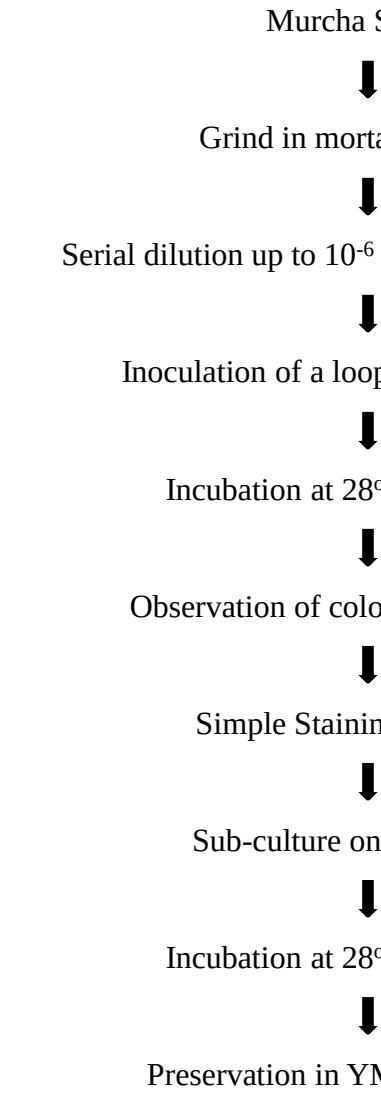
Flowchart 1: Screening of Fermentative yeast

3.3.4 Purification

An isolated colony of the selected yeast was streaked on sterile YMA plate. Each isolate was sub-cultured in duplicate plates. One for the stock culture and other for working culture. All the plates were properly marked with code and incubated at 28°C for 48 hours.

3.3.5 Preservation

The pure culture of yeast in YMA plates after proper incubation was stored in refrigerator at 4°C. They were examined at regular intervals for evidence of purity. The process of isolation of yeast from murcha is given in Flowchart 2.



Flowchart 2: Isolation and Preservation of Yeast Isolate

3.4 Screening for Thermotolerant Yeast

Temperature tolerance test was performed using Yeast Malt Agar. Fresh subculture of yeasts was streaked onto the agar plates. Incubation was done at different temperatures (25°C, 30°C, 37°C, 45°C and 50°C) for 48 hours. Tolerance was determined by observing the presence or absence of visible colonies on the agar plates (Guimarães et al., 2006).

3.5 Yeast Tolerance to pH

Tolerance to pH was observed in yeast using Yeast Malt Broth. A loopful of fresh subculture of yeast was inoculated into YMB tubes with 5 ml broth media at various pH levels ranging from 1 to 8. The high pH value was maintained by using 1M sodium hydroxide and low pH value was adjusted with the help of 1M hydrochloric acid (Hossain et al., 2020). pH was confirmed through use of pH meter. Observation of growth was conducted after incubation at 28^{o}C for 48 hours as visible turbidity in the medium.

3.6 Glucose Tolerance Test

Glucose tolerance test was performed using Yeast Malt Broth with different concentrations of glucose, 10%, 15%, 20%, 25%, 30%, 35%, 40% (m/v), respectively (Sree et al., 2000) in 5 ml media. Fresh subcultures were inoculated into the media tubes using an inoculating loop. The tubes were incubated for 48 hours at 28^{o}C. Growth was observed as cell density.

3.7 Screening for Ethanol Tolerance

Preliminary ethanol tolerance test was performed for all 8 yeast isolates and *S.cerevisiae*. This was done by inoculating a loopful of pure yeast culture into 5 ml Yeast Malt Broth in 8 ml test tubes containing ethanol in concentrations ranging from 0 to 20%. The tubes were incubated at 28^{o}C for 48 hours. Tolerance was determined by observing the presence or absence of visible surface growth and turbidity in the media (Ünaldi Coral et al., 2002).

3.8 Ethanol Tolerance Test

Ethanol tolerance test was performed using Yeast Malt Broth with different concentrations of ethanol, 0%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, 20% (v/v), respectively. Fresh subcultures were inoculated into the media tubes containing 5 ml broth media using an inoculating loop. The tubes were incubated for 48 hours at 28^{o}C. Results were observed at OD_{600} using a spectrophotometer (Ernandes et al., 1990).

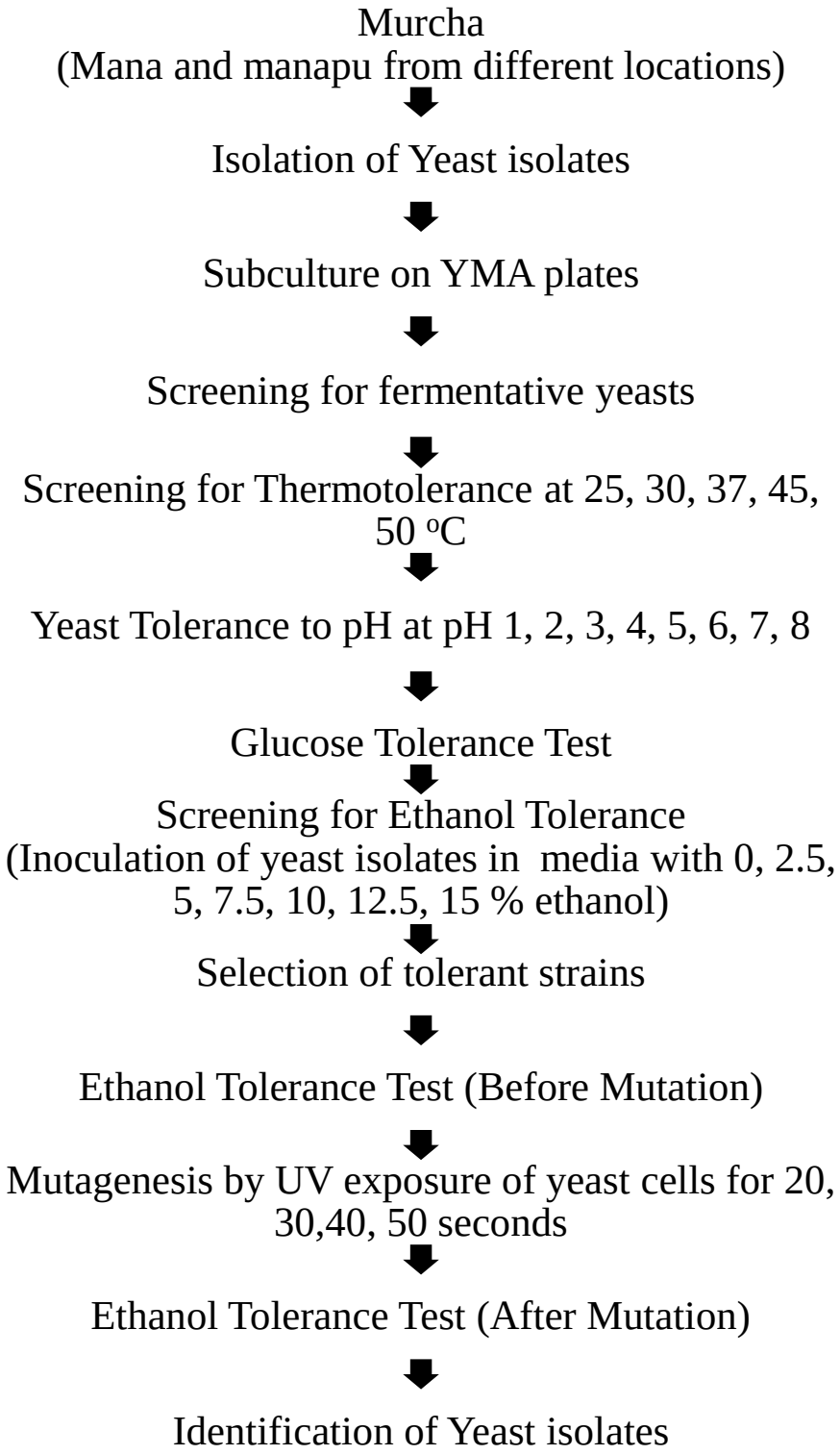
3.9 Mutagenesis

Mutagenesis was induced by using UV radiation. Yeast cultures obtained after 24 hours of incubation were inoculated on Yeast Malt Agar plates by streak plating method and were exposed to UV radiation in a UV chamber for 20, 30, 40, 50 seconds and incubated at 28^{o}C for 72 hours. After incubation, the alcohol tolerance tests were repeated (Ünaldi Coral et al., 2002).

3.10 Identification of yeast isolates

Identification was carried out by carbohydrates utilization test. The test was carried out in tubes with peptone water with phenol red as indicator, different carbohydrates; 1% (m/v) and inverted Durham's tube (Tsuyoshi et al., 2005). The yeast isolates were inoculated into the media tubes with 5ml media and incubated for 48 hours at 28^{o}C. Carbohydrates used were Arabinose, Raffinose, Sucrose, Maltose, Lactose, Galactose, Dextrose, Fructose, Xylose, and Glucose. Observation was done on the colour of the media after incubation and the gas production capacity. The sugars utilized gave insight on the species of the yeast isolates.

RESEARCH DESIGN



Flowchart 3: Overview of research design

Chapter IV

RESULTS

4.1 Sample description with isolated isolates and microbial load

Thirty different murcha samples (21 manapu and 9 mana) were collected from different parts of Nepal. The sample description with isolated isolates is given in Table 1. Altogether, 21 yeast isolates were isolated from 30 murcha samples. Enumeration of yeast is given in Table 2.

Table 1: Sample description with isolates

No. of sample	Sample code	Sample collected from	Yeast isolates
1	M1	Syangja	1
2	M2	Syangja	-
3	M3	Syangja	-
4	M4	Naikap	-
5	M5	Naikap	1
6	M6	Bhaktapur	-
7	M7	Bhaktapur	-
8	M8	Bhaktapur	2
9	M9	Bhaktapur	1
10	M10	Bhaktapur	2
11	M11	Bhaktapur	-
12	M12	Jiri	1
13	M13	Jiri	1
14	M14	Jiri	1
15	M15	Thecho	1
16	M16	Thecho	1
17	M17	Thecho	1
18	M18	Kathmandu	-

19	M19	Kathmandu	2
20	M20	Kathmandu	-
21	M21	Kathmandu	-
22	M22	Lamjung	-
23	M23	Lamjung	2
24	M24	Lamjung	1
25	M25	Lalitpur	2
26	M26	Lalitpur	-
27	M27	Lalitpur	-
28	M28	Lalitpur	1
29	M29	Chitwan	-
30	M30	Chitwan	-
Total	**30**		**21**

Table 2: Enumeration of yeast

No. of sample	Sample code	Sample collected from	Load of yeast (cfu/gm)
1	Y1	Syangja	2×10^7
2	Y2	Naikap	2.68×10^7
3	Y3	Bhaktapur	2.13×10^7
4	Y4	Bhaktapur	1.6×10^7
5	Y5	Bhaktapur	4.9×10^7
6	Y6	Bhaktapur	8.3×10^7
7	Y7	Bhaktapur	6.8×10^7
8	Y8	Jiri	1×10^8
9	Y9	Jiri	2.4×10^7
10	Y10	Jiri	1.3×10^7
11	Y11	Thecho	4.05×10^7
12	Y12	Thecho	6.7×10^7
13	Y13	Thecho	7×10^7

14	Y14	Kathmandu	4×10^8
15	Y15	Kathmandu	7.7×10^7
16	Y16	Lamjung	5.6×10^7
17	Y17	Lamjung	2×10^7
18	Y18	Lamjung	4.68×10^9
19	Y19	Lalitpur	10×10^7
20	Y20	Lalitpur	5.58×10^7
21	Y21	Lalitpur	8×10^9

4.2 Isolation of yeast from murcha sample

Twenty-one yeast colonies were isolated from murcha samples. Most of the yeast colonies were creamy in colour and circular. Size of the raised and convex colonies ranged from 1-3 mm in diameter. On microscopic examination after simple staining, all isolates were oval or circular. Budding was observed on most of the isolates. The observation is clarified in Table 3.

Table 3: Macroscopic and Microscopic Characteristics of yeast isolates

S.N	Isolate Codes	Macroscopic Observation	Microscopic Observation
1	Y1, Y2, Y3, Y4, Y6, Y7, Y15, Y18, Y19	Cream coloured, circular, 1-3mm, opaque, mucoid, raised, entire	Spherical cells, budding was seen
2	Y5, Y8, Y9, Y10, Y11, Y12, Y13, Y14, Y16, Y17, Y20, Y21	Creamy white colour, 2-3 mm, mucoid, raised, glossy, slightly convex, entire	Ovoid cells, budding was seen

4.3 Screening of Fermentative Yeasts

21 presumptive yeast isolates and *S.cerevisiae* were further screened for their fermentative ability on the basis of three parameters: cell growth, gas production (Durham's tubes) and alcohol production (sensory evaluation). Among the tested isolates, 2 yeast isolates (Y13 and Y17) were found to be non-fermentative i.e., non-gas and non-alcohol producer, 9 yeast isolates (Y1, Y2,

Y3, Y4, Y5, Y7, Y12, Y16 and Y18) were found to be only alcohol producer and 8 yeast isolates (Y8, Y9, Y10, Y11, Y14, Y15, Y19, Y20, Y21, and *S.cerevisiae*) were found to be both gas and alcohol producer. The highest fermentative ability was found in Y15 and Y21 whereas the lowest fermentative ability was observed in Y9. The fermentative ability of other isolates was in order of Y10, Y11, *S.cerevisiae* >Y19>Y20>Y14, Y8 (Table 4).

Table 4: Screening of fermentative yeasts

Isolate Code	**Gas production**	**Turbidity**	**Alcoholic Smell**
Y1	-	++++	+
Y2	-	++++	++
Y3	-	+++	++
Y4	-	++++	++
Y5	-	++++	+++
Y6	+++	+++	+
Y7	-	+++	+++
Y8	+++	+++	+
Y9	+	++++	++
Y10	++	+++	+++
Y11	++	++++	+++
Y12	-	++++	++
Y13	-	++++	-
Y14	+++	+++	+
Y15	+++	++++	+++
Y16	-	+++	+
Y17	-	+++	-
Y18	-	++++	++
Y19	++	++++	+++
Y20	+++	+++	++

Y21	+++	++++	+++
S.cerevisiae	++	++++	+++

+ = low +++ = high

++ = moderate ++++ = very high

4.4 Screening for Thermotolerant Yeast

Similarly, the temperature tolerance capacity of the 8 fermentative yeast isolates and *S.cerevisiae* were studied by observing the colony formation after incubation at different temperature (25° to 50°C). As seen in Table 5, 3 yeast isolates (Y8, Y9 and Y19) were seen to grow at up to 37°C and 5 isolates (Y10, Y11, Y15, Y20, Y21, and *S.cerevisiae*) were observed to grow at up to 45°C. None of the isolates were found to be growing at 50°C.

Table 5: Temperature Tolerance Test

Isolate Code	**Temperature**				
	25	30	37	45	50
Y8	+	+	+	-	-
Y9	+	+	+	-	-
Y10	+	+	+	+	-
Y11	+	+	+	+	-
Y15	+	+	+	+	-
Y19	+	+	+	-	-
Y20	+	+	+	+	-
Y21	+	+	+	+	-
S.cerevisiae	+	+	+	+	-

+ = Growth

- = No Growth

4.5 Yeast Tolerance to pH

Eight fermentative yeasts and *S.cerevisiae* were inoculated into media with different pH and the observation was done after incubation. As can be seen, most yeast isolates have the highest growth around pH 4. The growth decreases on both sides of the pH scale from this point. Isolates Y9 and Y21 were not observed to be growing at pH 8. At pH levels higher than 7, growth is observed to decline for all isolates. Growth of all isolates started declining on pH higher than 6. Optimum pH levels for yeast growth were observed to be from 3 to 5. *S.cerevisiae* was observed to have more tolerance towards the acidic pH levels.

Table 6: Yeast Growth at different pH

Isolate Code	**pH of medium**							
	1	2	3	4	5	6	7	8
Y8	+	+	++	+++	+++	++	++	+
Y9	+	+	+	+++	+++	++	++	-
Y10	+	+	+++	+++	+++	+++	++	+
Y11	+	+	+++	+++	+++	++	++	+
Y15	+	+	++	+++	+++	++	++	+
Y19	+	+	+++	+++	+++	++	++	+
Y20	+	+	++	+++	+++	++	++	+
Y21	+	+	++	+++	+++	++	++	-
S.cerevisiae	+	++	+++	+++	+++	+++	++	+

- = No Growth

+ $\geq$ 0.25 OD_{600}

++ = 0.25 - 0.5 OD_{600}

+++ = 0.5 - 0.75 OD_{600}

4.6 Screening for Glucose Tolerance

The yeast isolates and *S.cerevisiae* were then assayed for their glucose tolerance by measuring the cell growth in presence of 7 different concentrations of glucose (ranging from 10% to 40%). The glucose tolerance capacity was found

to be not significantly different in the tested yeast isolates. All tested isolates were observed as having very good cell growth in all concentrations of glucose.

Table 7: Screening for glucose tolerance

Isolate	**Glucose concentration % (m/v)**						
	10	15	20	25	30	35	40
Y5	++++	+++	++++	++++	++++	++++	++++
Y7	++++	+++	++++	++++	++++	++++	++++
Y10	++++	+++	++++	++++	++++	++++	++++
Y11	+++	+++	++++	++++	++++	++++	++++
Y15	++++	+++	+++	++++	++++	++++	++++
Y19	++++	++++	++++	++++	++++	++++	++++
Y20	++++	++++	++++	++++	++++	++++	++++
Y21	++++	+++	+++	++++	++++	++++	++++
S.cerevisiae	++++	+++	++++	++++	++++	++++	++++

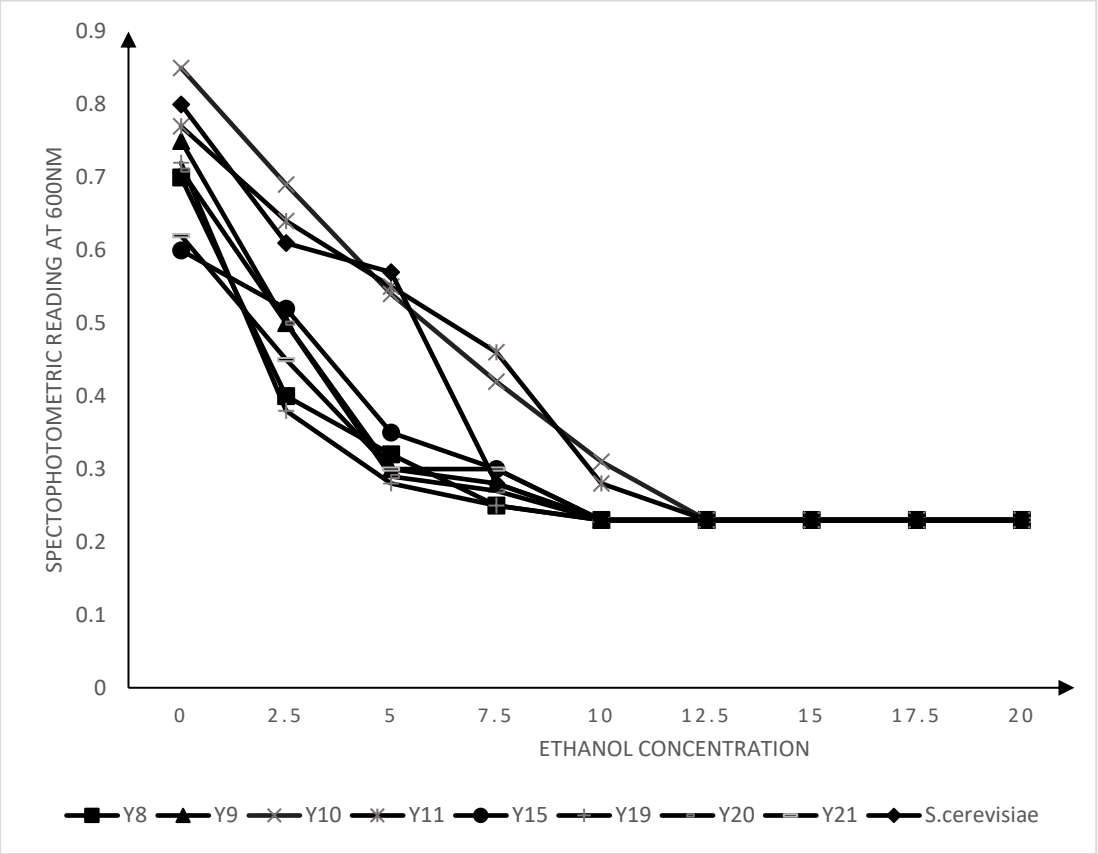
+++ = 0.5 – 0.75 $0D_{600}$

++++ = 0.75 – 1.00 OD_{600}

4.7 Screening for Ethanol Tolerance

Eight potential fermentative yeast isolates and *S.cerevisiae* were further assayed for their ethanol tolerance capacity by measuring the cell growth in presence of 9 different concentrations of ethanol (0%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5%, and 20%). The ethanol tolerance capacity was found to be significantly varied depending upon the yeast isolates. All the tested isolated showed reasonable cell growth in 5% ethanol. As expected, only a very few isolates had the ability to tolerate higher ethanol concentration. Only 2 yeast isolates (Y10 and Y11) showed tolerance up to 12.5%. All other yeast isolates including *S.cerevisiae* showed tolerance up to 10% (Appendix E). The trend of diminishing cell density with increase in ethanol concentration was seen in all

isolates as seen in Figure 5. The optical density observed in the control tube without any inoculation was observed to be 0.23.



OD_{600} reading for control => 0.23

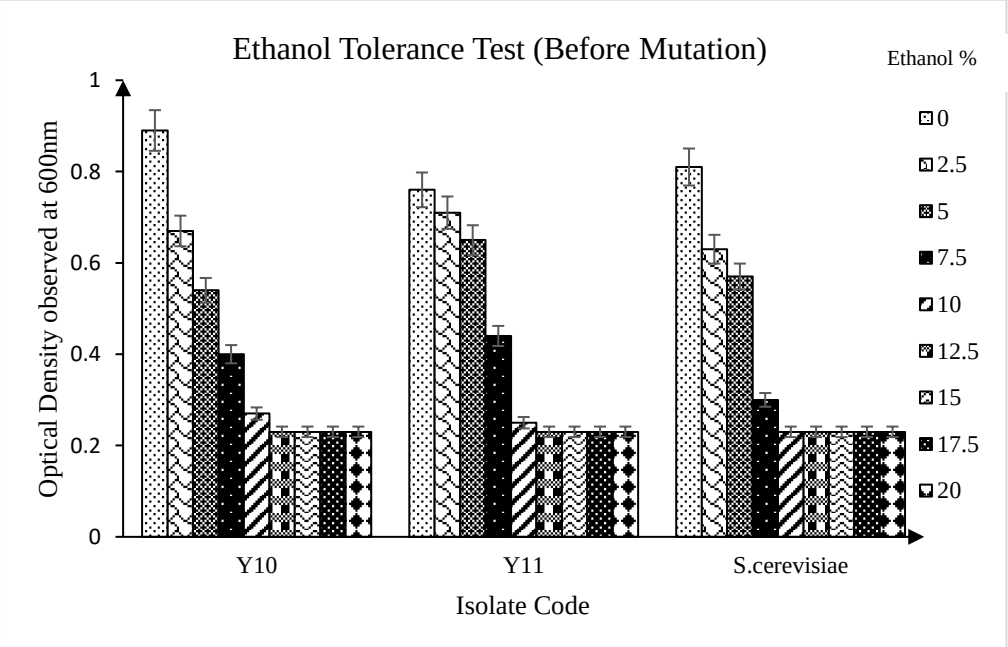
Figure 5: Screening for Ethanol Tolerance

4.8 Ethanol Tolerance Test

Based on the preliminary test for ethanol tolerance, the most growth in high percentages of ethanol was observed in isolated Y10 and Y11. These isolates were also among thermotolerant yeasts and had a wide range of pH tolerance. Isolates Y10 and Y11 were further tested for tolerating ethanol during fermentation along with *S.cerevisiae*. This was carried out before the UV treatment to establish a baseline and then after the irradiation process.

4.8.1 Before Mutation

Before mutagenesis, the isolate Y10 was not seen to tolerate ethanol above 7.5%. The cell density decline was observed from 0.89 to 0.54 (~39%) going from media with no ethanol to media with 5% ethanol. Isolate Y11's cell density decrease was observed as well but only from 0.73 to 0.65 (~11%). *S.cerevisiae* declined from 0.81 to 0.51 (~37%) going from normal media to media with 5% ethanol. This decreased further from 0.54 to 0.27 (~50%) in 10% ethanol for isolate Y10, from 0.51 to 0.26 (~49%) in 10% ethanol for isolate Y11, and from 0.57 to no growth at 10% ethanol for *S.cerevisiae* as seen in Figure 6. None of the isolates were able to grow above 10% ethanol (Appendix E). Baseline reading of 0.23 was observed in all isolates at ethanol percentages above 12.5%. Tolerance at 5% ethanol was highest in the isolate Y11 followed by *S.cerevisiae* and then lowest of the isolates tested in isolate Y10. Tolerance at 7.5% similarly was highest in isolate Y11, followed by Y10 and found to be lowest in *S.cerevisiae*.



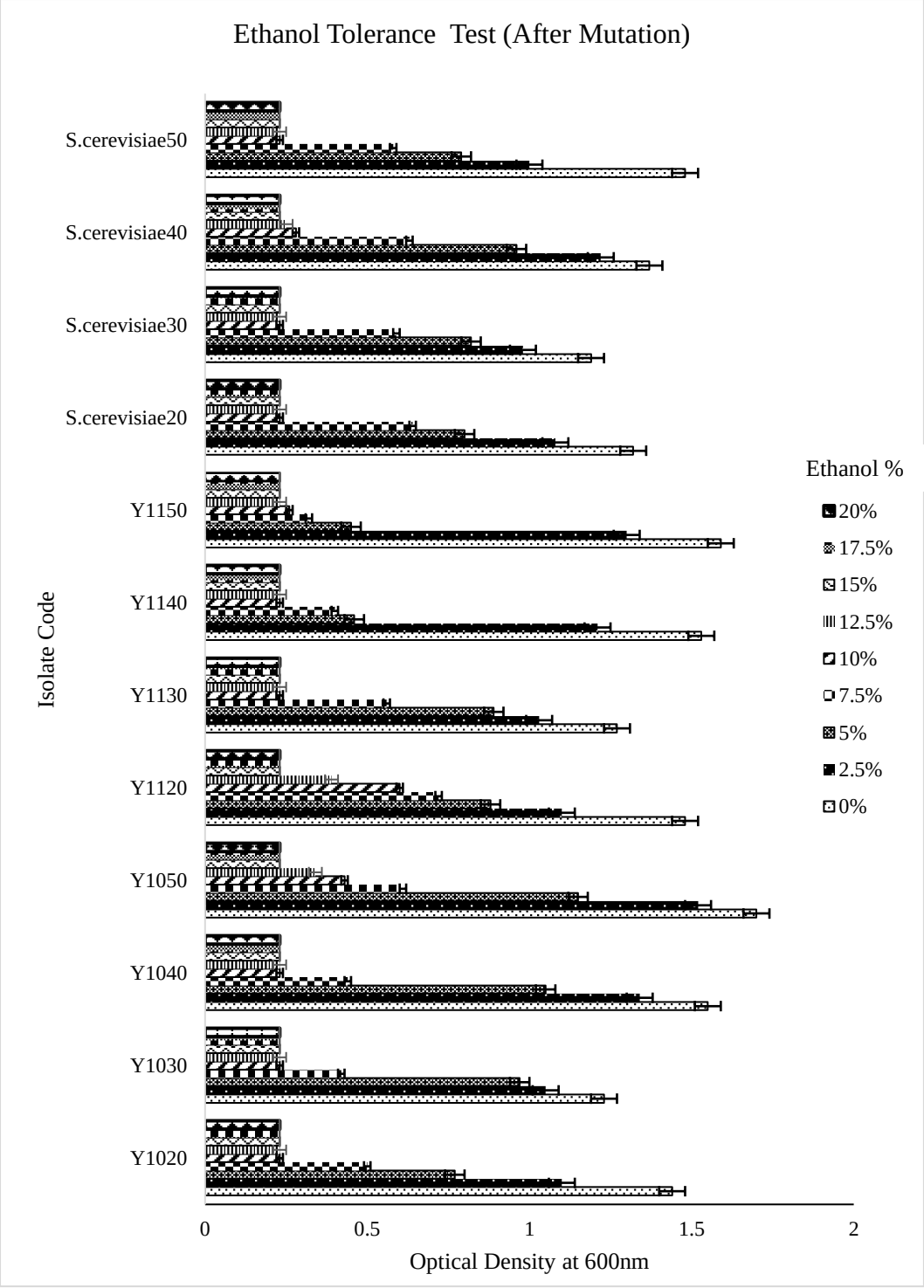
OD_{600} reading for control => 0.23

Figure 6: Ethanol Tolerance Test (Before Mutation)

4.8.2 After Mutation

After the isolates' baseline tolerance was measured, UV mutagenesis was conducted on the isolates. These mutated isolates were tested for their ethanol tolerance. Most of the mutated isolates were observed as having better growth at the same alcohol percentage including in the tubes with no alcohol. Isolate Y10 was found to have improved the most after 50 seconds in the UV chamber and Y11 had the most improved observation after 20 seconds. Five of the mutated isolates; $Y10_{40}$, $Y10_{50}$, $Y11_{20}$, $Y11_{50}$, and *S.cerevisiae* $_{40}$ were found to be able to grow at 12.5% ethanol (Appendix E). Most tolerance to ethanol was observed in isolate $Y11_{20}$ which had an OD of 0.6 at 10% ethanol and 0.39 at 12.5% ethanol. This was followed by $Y10_{50}$, which was found to have an OD of 0.43 at 10% ethanol and 0.34 at 12.5% ethanol, and *S.cerevisiae*$_{40}$, where the OD was observed at 10% to be 0.28 and at 12.5% to be 0.25 (Figure 7).

At the highest, the growth of isolate Y10 increased from 0.67 to 1.1 (~39%), the growth of Y11 was found to increase from 0.71 to 1.3 (~45%), and the growth of *S.cerevisiae* was seen as increasing from 0.63 to 1.22 (~48%) in 2.5% ethanol in the media.



OD_{600} reading for control => 0.23

Figure 7: Ethanol Tolerance Test (After Mutation)

4.9 Identification of yeast isolates

Isolates Y10 and Y11 were identified to potentially be *Saccharomyces* spp. Utilization was identified by development of yellow colour in the media along with gas trapped in the inverted Durham's tube. Isolate Y10 was observed as having developed yellow colour in tubes with Galactose, Dextrose, and Fructose. Gas was produced only in tube with fructose. Lactose was not utilized by both isolates. Isolate Y11 was found to fully utilize all sugars tested except Lactose and Xylose, the latter of which was partially utilized. Gas was produced in all tubes excluding the one with lactose. Tubes with Arabinose, Raffinose, Maltose for isolate Y10 and xylose for isolates Y10 and Y11 had orange colour and were observed as weak or partial utilization. The results were referenced with (Tsuyoshi et al 2005) for identification. The results of Y10 and Y11 were found to match with the results of the *S.cerevisiae* used in the test.

Table 8: Carbohydrate utilization test

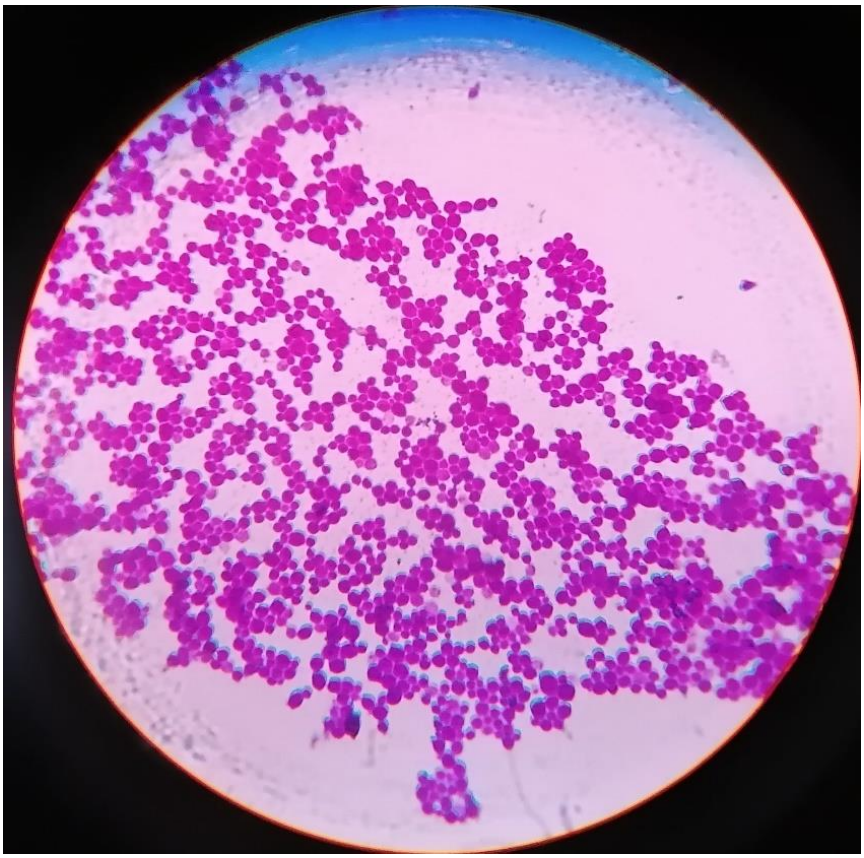
Isolate Code		**Carbohydrates**									**Infere-nce**
		Ara	Raf	Suc	Mal	Lac	Gal	Dex	Fru	Xyl	
Y10	Turbidity	Or	Or	+	Or	-	+	+	+	Or	*Saccharomyces spp.*
	Gas	-	-	-	-	-	-	-	++	-	
Y11	Turbidity	+	+	+	+	-	+	+	+	Or	
	Gas	+++	+++	+++	+++	-	+++	+++	+	+++	
S.cerevisiae	Turbidity	+	+	++	+	-	+	+	+	Or	*S.cere visiae*
	Gas	+	+	+	+	-	+	-	+	+	

+ = low ++ = moderate +++ = high or=weak

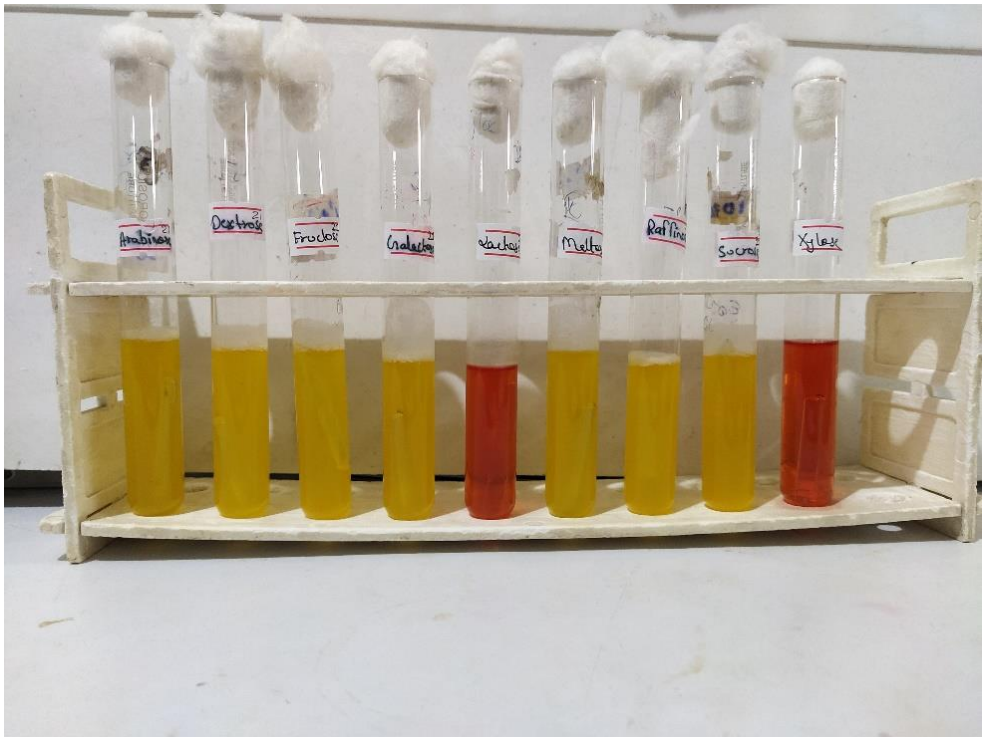
Ara= Arabinose Raf=Raffinose Suc=Sucrose Mal=Maltose Lac=Lactose Gal=Galactose Dex=Dextrose Fru=Fructose Xyl=Xylose

LIST OF PHOTOGRAPHS

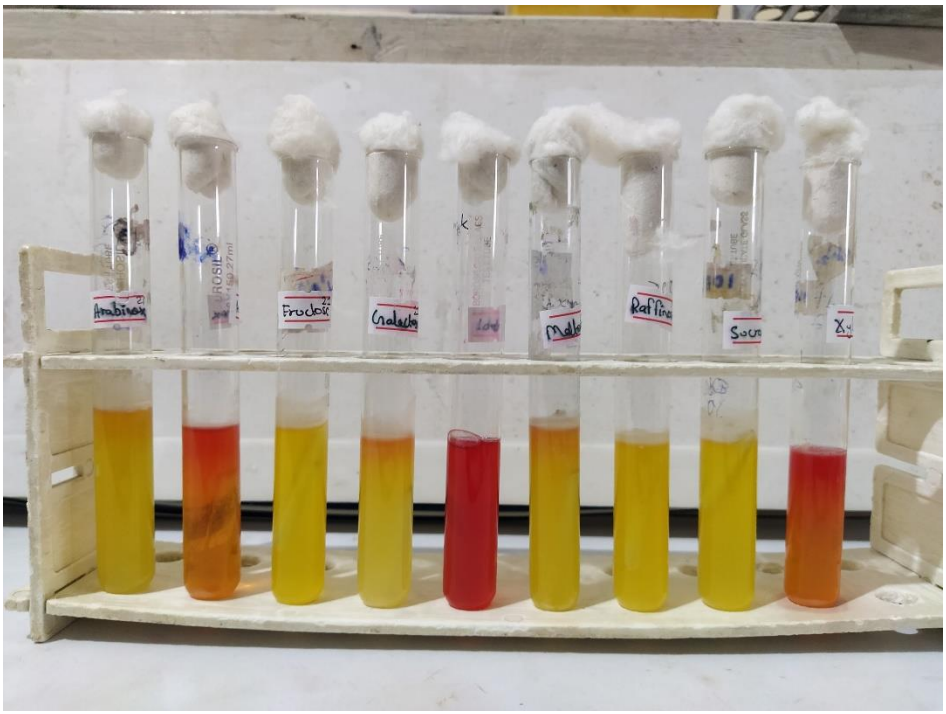
Photograph 1: Yeast isolates Y10 and Y11 on YMA plates



Photograph 2: Microscopic view of yeast cells



Photograph 3: Sugar Utilization test for identification of *Saccharomyces cerevisiae* (from left to right: Arabinose, Dextrose, Fructose, Galactose, Lactose, Maltose, Raffinose, Sucrose, Xylose)



Photograph 4: Sugar Utilization Test for identification of yeast isolate (from left to right: Arabinose, Dextrose, Fructose, Galactose, Lactose, Maltose, Raffinose, Sucrose, Xylose)

Chapter V

DISCUSSION

The utilization of yeasts in the production of alcoholic beverages represents one of the earliest biotechnological processes. Yeasts play a crucial role in the production of all alcoholic beverages, and the specific yeast isolates selected significantly impact both alcohol yield and sensory quality. Ethanol, or ethyl alcohol, is a chemical compound with a hydroxyl group attached to an ethane molecule, produced both chemically and biologically. Industrial ethanol is usually chemically produced, while consumable alcohol is biologically produced through the fermentation of carbohydrates from crops like corn, sugarcane, rice, and grains (Coelho et al., 2008). Widely used in industrial and consumer products, ethanol serves as a solvent and an intermediate in producing chemicals, drugs, plastics, cosmetics, and lacquers. It is used in drugs as an antiseptic and methanol poisoning antidote, and in non-beverage products like perfumes, colognes, mouthwashes, and aftershaves (Strohm, 2014).

Industrial-scale ethanol production may be chemical or biological. While lab grade ethanol is generally produced chemically by ethylene hydration (Weissermel & Arpe, 2003), food-grade ethanol production primarily uses yeasts, eukaryotic unicellular fungi found in soil, to ferment sugars. While some bacteria produce ethanol, yeasts are preferred due to their efficiency. Yeast strains and populations are influenced by environmental factors like temperature and pH. Yeasts have long been exploited for producing alcoholic beverages and bread (Phaff et al., 1966). Yeast strains are selected for fermentation based on their efficiency, with tolerance to temperature, pH, sugar, and ethanol being crucial factors to reduce production costs and improve yield (Gray, 1941).

Thirty murcha samples were collected in sterile collection bags from different locations of Nepal for this study. From these samples, 21 isolates were obtained and were identified as yeast by studying cultural and morphological features as described in Introduction to Fungal Physiology (Walker and White, 2017). They were seen to be white or cream coloured, mucoid, opaque, raised, slightly

convex, with entire margin. The ovoid cells were seen with buds under a microscope.

On screening of these isolates for ethanol production, 9 isolates including the *S.cerevisiae* utilized the glucose in the media and metabolized it into ethanol and carbon dioxide. This was observed as alcoholic smell from the tubes and accumulation of gas in the inverted Durham's tubes (Guimarães et al., 2006).

The isolates were tested for their respective temperature tolerances. The isolates were inoculated on YMA plates by spread plate method and incubated at different temperatures. 3 of the isolates (Y8, Y9, Y19) were found to grow at 25-37^{o}C and the other 6 (Y10, Y11, Y15, Y20, Y21, *S.cerevisiae*) at 25-45^{o}C. The isolates that can tolerate 45^{o}C are known as thermotolerant yeasts and are considered to be more efficient in ethanol production. This result is backed in Ernandes et al. (1990), where growth of yeast cells and ethanol production was higher at 30^{o}C than at 25^{o}C for the yeast cells. Similarly, Navarro and Durand (1978) reported ethanol accumulation in yeast cells increased with increase in temperature. The yeasts used in the experiment were isolated from murcha where the environment is dry, and the storage conditions are sub-optimal. The result may be due to change that happened in the murcha as stress response of the yeast (Crawford and Pavitt, 2019). However, this result is very different from Benítez et al., 1983 observations where growth and metabolism decrease with increase in temperature, which point towards the yeast cells not being thermotolerant in the experiment.

The isolates were tested for their pH resistance. Inoculation was done in media tubes set at different pH levels and growth was observed visually after incubation. All isolates were found to have the same pattern of growth. At pH 1 and 2, low turbidity was observed which signified minimal growth. Higher growth was observed in pH 3 to 5 after which the growth subsided enough that two isolates Y9 and Y21 had no growth at pH 8. This result is consistent with the results in Hossain et al. (2020), where the same growth pattern of increased growth at pH 4 and decreasing growth was observed at pH higher than 4 and pH lower than 4. This phenomenon is explained in part by the cell structure of yeast.

Yeasts have thicker cell envelope than non-acid tolerant microorganisms which may lead to acid-tolerance (Nguyen et al., 2001). In addition, yeast proteins are optimized for high acid environments since most yeasts are found naturally in acidic environments (Buzzini et al., 2018).

Eight isolates from ethanol screening and *S.cerevisiae* were then tested for glucose tolerance. The isolates were inoculated into media tubes with different concentrations of glucose. All isolates had maximum biomass production at 30% glucose before and after which the biomass depleted. The result is consistent with previous recordings Aldous et al. (1950), wherein the respiration rate of yeast cells increases up till a certain concentration of sugar after which it falls. The concentration was found to be 30% for most isolates and 35% at the highest, after which the biomass decreases. This however is inconsistent with results obtained by Bryan et al. (2018) where the biomass is highest at 80% sugar concentration after which it decreases.

These isolates were then subjected to preliminary ethanol tolerance test. The isolates were inoculated into YMB tubes with 9 different percentages of ethanol (0, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20) and incubated. After incubation, the tubes were visually inspected for growth. This test confirmed that isolates Y10 and Y11 were the most tolerant of ethanol (Chen et al., 2017) in the media as they grew at up to 12.5% ethanol while the others including *S.cerevisiae* stopped growth at 10%. This result is consistent with the one in Tikka et al. (2013) and Benítez et al. (1983) where the tolerance ranged from 7-12% with more organisms at the lower end of the range tolerating about 8% ethanol while some outlying yeast strains were observed to be growing at 12% ethanol. According to Sahana et al. (2024), wild yeasts tolerate around 8% ethanol and industrial strains tolerate up to 16% in some cases. This is in contrast to the *S.cerevisiae* tested from the brewery. This may mean that industrial microbes are highly improved and developed for the purpose of fermentation, but local breweries may be using non-industrial fermentative yeasts as industrial yeast strains are often expensive to procure.

Isolates Y10 and Y11 and the *S.cerevisiae* were observed as being thermotolerant from earlier tests. These isolates also were found to have a higher pH range in which they were seen as growing. Since thermotolerant yeasts are seen as having a greater ethanol yield and are not negatively impacted by higher temperatures (Ernandes et al., 1990), isolates Y10, Y11, and *S.cerevisiae* were subjected to a baseline ethanol tolerance test before undergoing mutation as these two were the most tolerant of ethanol in the preliminary test. Growth observed as optical density of media after incubation was 0.67, 0.54, 0.4, 0.27 at 2.5%, 5%, 7.5%, 10% ethanol respectively for isolate Y10. Optical density of media for isolate Y11 was found to be 0.71, 0.65, 0.44, 0.25 at 2.5%, 5%, 7.5%, 10% ethanol respectively. The readings at 2.5%, 5%, 7.5% ethanol for *S.cerevisiae* were 0.63, 0.57, 0.3 respectively. After the baseline tests were done, the isolates were then subjected to UV radiation. This was done by inoculating the yeast isolates on YMA plates by streaking and exposing to UV radiation for different time periods (20s, 30s, 40s, 50s). After mutation, the ethanol tolerance test was repeated. Across both isolates tested, there was an increase of 40-60% biomass in media with 2.5% ethanol. All isolates were able to tolerate 7.5% before mutation which increased to 12.5% for all isolates after mutation. The OD observed increased from 0.27 to 0.43 at 10% ethanol, 0.23 which was the baseline reading for the control solution to 0.34 at 12.5% ethanol for isolate Y10. Similar increase from 0.25 to 0.72 and 0.23 to 39 was observed at 10% and 12.5% ethanol for isolate Y11. The OD values for *S.cerevisiae* was found to have increased from 0.23 to 0.28 and 0.23 to 0.25 at 10% and 12.5% respectively. The result is consistent with research conducted by Neelam & Amarjit (1991) in which the ethanol tolerance of yeasts increased to 11.5% from 9.5%. Similar reports can be seen in Ünaldi Coral et al. (2002)'s research on yeast strain improvement by UV mutagenesis. The exact process of improvement of growth in this manner has not yet been accurately determined but this may be explained as a result of upregulations in genes related to vacuolar function, transcription, or mitochondrial function among other genes (Auesukaree et al., 2009). Likewise, ethanol stimulates synthesis of the protein Hsp12. This protein is located in the membrane and provides ethanol tolerance to the cells (Sales et al., 2000). Since ethanol tolerance is regulated by more than

250 genes (Boulton et al., 1999), the exact gene that could have caused the increase in tolerance needs to be studied by more vigorous means.

Tolerance seen was lower in *S.cerevisiae* than the yeasts isolated from murcha. The yeasts in murcha are not maintained properly and the flora of murcha as well as the fermentation substrate is not standardized. Storage conditions may lead to sporulation as a result of which the characteristics of yeast cell changes (Speers and Forbes, 2015) and the change may have occurred in the tolerance genes. However, commercial *S.cerevisiae* obtained from brewery has been maintained properly and was obtained from the wort of a brew. The brewery process is standardized and as such, there is little chance of yeast cells undergoing anything other than vegetative reproduction. It is also likely that the yeast strain obtained from the brewery was not industrialized and the brewery had been using wild fermentative yeast.

Additionally, the growth increased in the media without any ethanol as well. The growth was from 0.89 to 1.7 for isolate Y10, 0.73 to 1.59 for Y11, and 0.81 to 1.48 for *S.cerevisiae*. As discussed above, ethanol tolerant genes are closely related to membrane function. Additionally, there are genes that involve metabolism, cell cycle, DNA processing, cell defense, and protein synthesis that confer ethanol tolerance which may increase growth rate in the absence of stress. Furthermore, earlier studies have shown that some mechanisms required for ethanol tolerance may be required for thermotolerance. In addition to heat tolerance, there is significant overlap between ethanol tolerance and generally high osmotic stress response. Genes providing tolerance to ethanol have been observed inducing defense against antioxidants including production of reactive oxygen species in mitochondria (Auesukaree et al., 2009).

Identification was carried out by carbohydrate utilization test. Both isolates utilized their respective sugars, both were lactose non-fermenters, which on comparison with results obtained from previous studies; non-utilization of lactose, partial utilization of xylose and complete use of maltose, sucrose, arabinose, galactose, dextrose, raffinose, and fructose (Tsuyoshi et al., 2005) indicates the yeast isolates Y10 and Y11 to be *Saccharomyces* spp.

These results provide evidence that strain improvement of yeasts in respect to ethanol tolerance is possible at a low enough cost. The isolates Y10 and Y11 may be used as a fermenter which helps in avoiding stalling effect for longer and also provide a more efficient process. The fermentation temperature and pH can fluctuate without impacting ethanol production in the fermenter. Improvement of thermotolerant industrial yeast strains may produce even more efficient and robust yeasts that can be used in conjunction with other fermentation improvement methods for cost-effectiveness in production.

Renewable transportation fuels, like bioethanol, play a crucial role in mitigating global climate change. Bioethanol, primarily derived from biomass fermentation by yeast, provides a sustainable alternative to fossil fuels. By reducing greenhouse gas emissions and improving energy security, it offers a promising solution. However, the industrial production of bioethanol encounters obstacles, notably the toxicity of ethanol to the yeast *Saccharomyces cerevisiae*, which restricts ethanol yield and productivity (Bai et al., 2004). The complexity of ethanol tolerance in *S.cerevisiae* arises from a combination of genetic and environmental factors. Despite extensive research, no individual genetic modification has been identified to substantially enhance ethanol tolerance at high concentrations. Consequently, the optimization of yeast strains through mutation and other genetic techniques remains crucial for advancing bioethanol production technologies (Lam et al., 2014). The stalling effect seen in metabolism processes is an obstacle. Widespread industrial production of ethanol using yeast needs to be simple and efficient and strain improvement is one of the methods of doing this along with process optimization, environment and substrate improvements.

The development of yeast strains with enhanced ethanol tolerance can significantly improve the efficiency and cost-effectiveness of bioethanol production (Ali, 2014). Benefits include increased ethanol yield (Maharjan et al., 2013), improved fermentation kinetics, reduced inhibition (Ünaldi Coral et al., 2002), and economic and environmental impact. Tolerant isolates can survive and produce ethanol at higher concentrations, leading to higher overall yields. Enhanced tolerance can reduce lag phases and increase fermentation

rates, shortening production times. Strains that can withstand higher ethanol concentrations may also be less susceptible to inhibition by other fermentation by-products. Improved yeast strains can lower production costs, making bioethanol more competitive with fossil fuels and reducing the carbon footprint of transportation fuels (Lam et al., 2014).

Chapter VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

Microbial fermentation has long been employed for the efficient synthesis of various organic compounds, either for consumption or for use in food production. Among these processes, yeast-mediated production of food-grade alcohol from sugar is perhaps the most widely recognized. In recent years, research focus has shifted from fine-tuning reaction parameters to enhancing the performance of the microorganisms themselves. While precise strain improvement through gene manipulation is an option, it can be cost-prohibitive. Alternatively, a simpler approach involves inducing mutations in the microorganisms. In this investigation, our primary objective was to isolate and identify fermentative yeast from an indigenous fermenter, followed by enhancing alcohol tolerance through UV-induced mutations. Among 30 murcha samples tested, 21 isolates were obtained. These isolates resembled yeast by observing the cultural and morphological characteristics. Eight isolates coded as Y5, Y7, Y10, Y11, Y15, Y19, Y20 and Y21 were able to ferment sugar to produce ethanol when grown on Yeast Malt Broth and incubated at 28^{o}C for 48 hrs. Among these isolates, Y10 and Y11 were able to tolerate the most ethanol during screening. The tolerance was quantified by observing the growth at OD_{600} using a spectrophotometer after inoculation in media supplemented with alcohol and incubation. Mutation was carried out under UV light and the ethanol tolerance test was repeated. The mutated isolates, namely, $Y10_{50}$ and $Y11_{20}$ were found to have increased tolerance to ethanol in terms of ethanol percentage while all mutated isolates were found to have more growth in the media than the unmutated isolates.

6.2 Recommendations

1. Quantitative values of ethanol produced by fermentation in the media should be determined.
2. Further research should be done to accurately determine the genes in *Saccharomyces* spp. responsible for the increase in ethanol tolerance.
3. The exact cause of increased biomass in mutated yeast isolates in media without ethanol may be researched.
4. Industrialization of mutated isolates of yeast for a more efficient alcohol production.

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APPENDIX A

LIST OF EQUIPMENT AND MATERIALS USED DURING THE STUDY

A. Equipment

Weighing machine

Mortar and pestle

Incubator

Hot air oven

Refrigerator

Autoclave

Microscope

Spectrophotometer

UV lamp chamber

B. Microbiological Media

Yeast Malt Agar

Yeast Malt Broth

Peptone water

C. Chemicals and Reagents

Hydrochloric acid

Sodium hydroxide

Ethanol

Phenol Red

Glucose

Arabinose

Raffinose

Sucrose

Maltose

Lactose

Galactose

Dextrose

Fructose

Xylose

Glycerol

D. Miscellaneous

Conical flask, Cotton, Distilled water, Glass slides, Immersion oil, inoculating loop, Measuring cylinder, Petri dishes, Pipettes, Spatula, Eppendorf's tube, Test tubes, Bunsen burner

APPENDIX B

COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

The culture media were from Hi-Media laboratories Pvt. Limited, Mumbai, India.

1. Yeast Malt Agar

	Grams/Litre
Yeast Extract	3
Malt extract	3
Peptone (Bacteriological).	1
Glucose	2
Agar	15
Distilled water	1000ml
Final pH	4.5 – 5.0 ± 0.2

Directions: The components of the media were weighed individually and mixed in 1000 ml distilled water, taken in a conical flask. It was boiled to dissolve completely and capped using a cotton plug. The media was sterilized by autoclaving at 15 lbs. pressure at 121˚C for 15 minutes. After cooling the media to about 50˚C, 20 ml media was poured into sterile petri plates and left undisturbed to solidify.

Tartaric acid was added to the media to lower the pH if necessary.

2. Yeast Malt Broth

Ingredients	Grams/Litre
Yeast Extract	3
Malt extract	3
Peptone (Bacteriological).	1
Glucose	2
Distilled water	1000ml
Final pH	4.5 – 5.0 ± 0.2

Direction: The components of the media were weighed individually and mixed in 1000 ml distilled water and boiled to dissolve completely and

dispensed into screw capped tubes. The tubes were sterilized by autoclaving at 15 lbs. pressure at 121˚C for 15 minutes.

3. Peptone Broth

Ingredients	Grams/Litre
Peptone	10
Sodium chloride	5
Phenol Red	0.018
Carbohydrate	10
Distilled water	1000ml
Final pH	7.2 ± 0.2

Directions: 15 g of peptone water was weighed out and dissolved in 1000 ml distilled water. 0.018 g of phenol red and 10 g of the test carbohydrate was weighed out and dissolved completely. This mixture was dispensed in tubes with inverted Durham’s tubes and sterilize by autoclaving at 121°C for 15 minutes.

APPENDIX C

SIMPLE STAINING PROCEDURE

Simple staining

The following steps are involved in simple staining:

1. A thin and uniform smear or film of the yeast to be examined was prepared on a clean and grease free glass slide and air dried.
2. The smear was then heat fixed and allowed to cool.
3. The slide was flooded with crystal violet or cotton blue stain and allowed to remain on the surface for one minute, not letting the smear dry.
4. The slide was air dried and examined microscopically under oil immersion of 100X.

APPENDIX D

COMPOSITION AND PREPARATION OF TEST REAGENTS

Crystal Violet Stain

Ingredients	Grams/litre
Crystal violet	20.00
Ammonium oxalate	9.00
Ethanol or methanol	95 ml

Directions: In a clean piece of paper, 20 grams of crystal violet was weighed and transferred to a clean brown bottle, Then, 95 ml of ethanol was added and mixed until the dye was completely dissolved and to the mixture, 9 grams of ammonium oxalate crystals dissolved in 200 ml distilled water was added. Finally, the volume was made 1 litre by adding distilled water.

Glucose Stock

Ingredients	Mass/Volume
Glucose	50g
Distilled water	100 ml

APPENDIX E

Table : Screening for ethanol tolerance

Isolate Code	Ethanol Concentration % (v/v)								
	0	2.5	5	7.5	10	12.5	15	17.5	20
Y8	0.7	0.4	0.32	0.25	0.23	0.23	0.23	0.23	0.23
Y9	0.75	0.5	0.3	0.28	0.23	0.23	0.23	0.23	0.23
Y10	0.85	0.69	0.54	0.42	0.31	0.23	0.23	0.23	0.23
Y11	0.77	0.64	0.55	0.46	0.28	0.23	0.23	0.23	0.23
Y15	0.6	0.52	0.35	0.3	0.23	0.23	0.23	0.23	0.23
Y19	0.72	0.38	0.28	0.25	0.23	0.23	0.23	0.23	0.23
Y20	0.71	0.5	0.29	0.27	0.23	0.23	0.23	0.23	0.23
Y21	0.62	0.45	0.3	0.3	0.23	0.23	0.23	0.23	0.23
S.cerevisiae	0.69	0.48	0.32	0.28	0.23	0.23	0.23	0.23	0.23

Table: Ethanol Tolerance Test (Before Mutation)

Isolate Code	**Spectrophotometric reading at OD_{600} for ethanol concentration %(v/v)**								
	0	2.5	5	7.5	10	12.5	15	17.5	20
Y10	0.89± 0.03	0.67± 0.03	0.54± 0.03	0.4±0.03	0.27±0.01	0.23± 0.01	0.23± 0.01	0.23± 0.01	0.23± 0.01
Y11	0.76± 0.02	0.71± 0.03	0.65± 0.03	0.44± 0.03	0.25±0.01	0.23± 0.01	0.23± 0.01	0.23± 0.01	0.23± 0.01
S.cerevisiae	0.81± 0.02	0.63± 0.03	0.57± 0.02	0.3±0.03	0.23±0.01	0.23± 0.01	0.23± 0.01	0.23± 0.01	0.23± 0.01

Table: Ethanol Tolerance Test (After Mutation)

Isolate Code	**Spectrophotometric reading at OD_{600} for ethanol concentration %(v/v)**								
	0	2.5	5	7.5	10	12.5	15	17.5	20
$Y10_{20}$	1.44±0.04	1.1±0.04	0.77±0.04	0.5±0.04	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01
$Y10_{30}$	1.23±0.04	1.05±0.04	0.97±0.04	0.42±0.04	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01
$Y10_{40}$	1.55±0.04	1.34±0.04	1.05±0.04	0.44±0.04	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01
$Y10_{50}$	1.7±0.04	1.52±0.04	1.15±0.04	0.61±0.04	0.43±0.03	0.34±0.01	0.23±0.01	0.23±0.01	0.23±0.01
$Y11_{20}$	1.48±0.04	1.1±0.04	0.88±0.04	0.72±0.04	0.6±0.03	0.39±0.01	0.23±0.01	0.23±0.01	0.23±0.01
$Y11_{30}$	1.27±0.04	1.03±0.04	0.89±0.04	0.56±0.04	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01
$Y11_{40}$	1.53±0.04	1.21±0.04	0.46±0.04	0.4±0.04	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01
$Y11_{50}$	1.59±0.04	1.3±0.04	0.45±0.04	0.32±0.04	0.26±0.02	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01
***S.cerevisiae*$_{20}$**	1.32±0.04	1.08±0.04	0.8±0.04	0.64±0.04	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01
***S.cerevisiae*$_{30}$**	1.19±0.04	0.98±0.04	0.82±0.04	0.59±0.04	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01
***S.cerevisiae*$_{40}$**	1.37±0.04	1.22±0.04	0.96±0.03	0.63±0.04	0.28±0.02	0.25±0.01	0.23±0.01	0.23±0.01	0.23±0.01
***S.cerevisiae*$_{50}$**	1.48±0.04	1±0.04	0.79±0.04	0.58±0.04	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01	0.23±0.01