COLLEGE OF TECHNOLOGY AND BUILT ENVIRONMENT

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DEPARTMENT OF ENVIRONMENTAL ENGINEERING

OPTIMIZATION OF YEAST PROPAGATION AND MOLASSES FERMENTATION FOR ENHANCED ETHANOL PRODUCTION AND WASTE MINIMIZATION: THE CASE STUDY OF BALEZAF ALCOHOL AND LIQUOR FACTORY

A thesis is submitted to Addis Ababa University Addis Ababa Institute of Technology School of Chemical and Bio Engineering in partial fulfillment of the requirements for the Degree of Master of Science in Environmental Engineering.

BY

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Title: Optimization of Yeast Propagation and Molasses Fermentation for Enhanced Ethanol Production and Waste Minimization: The Case Study of Balezaf Alcohol and Liquor Factory

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Declaration

I, therefore, state that this thesis, "Optimization of Yeast Propagation and Molasses Fermentation for Enhanced Ethanol Production and Waste Minimization at Balezaf Alcohol and Liquor Factory", is my own work and has not been submitted in any form to any other institution or university for the award of any academic degree or diploma. All sources of materials used in the preparation of this thesis have been duly credited. Any help received throughout the process of this work has been ascertained and acknowledged. This thesis is presented to the Addis Ababa University, College of Technology and Built Environment, School of Chemical and Bio Engineering, in partial fulfillment of the requirements for the Degree of Master of Science in Environmental Engineering.

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Table of Contents

List of Tables

List of Figures

List of Abbreviations and Acronyms

AFC – Agricultural and Food Chemistry

BALF – Balezaf Alcohol and Liquor Factor

BBD – Box-Behnken Design

BOD – Biochemical Oxygen Demand

$CaCO_3$ – Calcium Carbonate

CaO – Calcium Oxide

CO_2 – Carbon Dioxide

COD – Chemical Oxygen Demand

DAP – Diammonium Phosphate

EDTA – Ethylene Diaminetetra Acetic Acid

FAO – Food and Agricultural Organization

HCl – Hydrochloric Acid

ICUMSA – International Commission for Uniform Method of Sugar Analysis

NaOH – Sodium Hydroxide

NPS – Nitrogen Phosphorus Sulfur Fertilizer

PPM – Part Per Million

RSM – Response Surface Methodology

SDGs – Sustainable Development Goals

VVM – Volumes of Air per Volume of Liquid per Minute

WHO – World Health Organization

Abstract

International demand for renewable energy sources has established research into the production of bioethanol at the highest possible levels from agro industrial wastes such as molasses. Maximal production of ethanol from molasses and minimization of waste output to the environment by optimization of yeast growth and molasses fermentation are the prime objectives of this research at Balezaf Alcohol and Liquor Factory (BALF), Ethiopia. Chemical analysis showed 46.02% fermentable sugars and 49.52% reducing sugars in raw molasses. Process conditions, i.e., pH, sugar concentration, temperature, dosage of nutrient, and inoculum of yeast, were optimized by Response Surface Methodology (RSM) and Box-Behnken Design (BBD). The optimum propagation conditions (pH 4.75, 1.0 g/L DAP + urea, and 1.5 g/L yeast) yielded 0.8% ethanol and 3.52×10^8 cells/mL cell count, and optimized fermentation (17% sugar, 30°C, 10% inoculum) yielded 12.1% ethanol with a meager 0.7% residual sugar far better than BALF's customary yields of 6.5–9.5% ethanol and 2–3.5% residual sugar. Recyclable yeast yielded 10.9% ethanol, an enviably rosy reading for cost efficient and eco-friendly production. ANOVA statistical test and model fit ($R^2 > 0.98$, $p < 0.05$) ensured the process reliability. The current study demonstrates the very first complete and scalable optimization method in BALF, an environmentally friendly model of bioethanol production in Ethiopia.

Keywords: Fermentation of molasses, Yeast recycling, Yeast propagation, Response Surface Methodology, Residual sugar, Minimization of waste, Balezaf Alcohol and Liquor Factory.

Chapter One

1. Introduction

1.1. Background of the Study

Ethanol production from molasses has also come into focus because the world is required to discover other alternative renewable sources to replace fossil fuels. Molasses is a secondary product of the sugar industry in the form of concentration and is filled with fermentable sugars like sucrose, glucose, and fructose (Hawaz et al., 2023). Production of ethanol through molasses is not only economically but even environmentally supportive on the basis of availability as well as affordability.

Fermentation is a pivotal phase in the process of conversion of sugar to ethanol and is succeeded by a chain of intricate biochemical reactions catalyzed by yeast. The yield/effectiveness determining factors vary from the conditioning of raw material to the preparation and selection of yeast, fermentation conditions, and the availability of nutrients. Conditioning is needed for molasses to remove impurities and enhance yeast activity. Operations such as dilution, filtration, and adding nutrients can have a significant impact on fermentation (Ruhul et al., 2013). This is due to the fact that where there are impurities; they will interfere with yeast activity, hence resulting in decreased ethanol yields. Also, the selection of yeast strain determines success in fermentation. Recycling and culturing are also some of the methods to manage yeast costs and optimize fermentation rates. Once they get in, the business has to be kept under ideal conditions. Temperature, pH, and oxygen levels are parameters that need to be monitored strictly. The ideal temperature for growth in yeast is typically 25-30ºC, and pH has to be tightly regulated between 4.5-5.5 (Salihu et al., 2023; Dasgupta et al., 2014). Below this, the fermentation process is weak and leads to a low yield of sugar per ethanol. Nutrients have to be supplemented to facilitate yeast growth and metabolism. Nitrogen sources are some of the most significant conditions that are used to activate yeast activity; the addition of urea or ammonium salts has demonstrated a statistically significant rate increase in fermentation yields (Rojo et al., 2023).

After fermentation is achieved, distillation is used to clean the ethanol. Distillation isolates ethanol from the broth of fermentation through the use of heat and condensation and as a result concentrates ethanol. Distillation is energy-poor and thus necessitates the need to look

for more efficient technologies and processes (Garg et al., 2023). Second, the process of distillation can generate big amounts of effluent distillery with high concentrations of organic pollutant.

Balezaf Alcohol and Liquor Factory is utilized in this case study to determine the practices and challenges of molasses fermentation and ethanol production industries. The factory has the challenge of achieving maximum ethanol productivity while minimizing effluent production effect on the environment (Balf, 2024). Based on experience at Balezaf, it is hoped through this study that best practices and innovative methods in achieving maximum efficiency in fermentation as well as sustainability will be determined.

Therefore, optimization of molasses fermentation for ethanol production is sophisticated and involves strict attention to raw material preparation, yeast management, fermentation conditions, and treatment of effluent.

1.2. Statement of the Problem

Ethanol production at Balezaf Alcohol and Liquor Factory (BALF) is presently limited by low fermentation efficiency and environmental cost. Despite molasses being very rich in nutrients and the fact that there is already existing fermentation equipment, the old traditional process of the factory only results in 6.5–9.5% ethanol with residual sugar content up to 2–3.5% (Balf, 2024). This not only leads to the loss of useful fermentable sugars but also produces high strength organic effluents, which considerably pose a threat to the sustainability of the environment.

Among the key operational issues is the absence of systematic optimization of yeast propagation and fermentation parameters. Parameters like pH, concentration of sugar, nutrient level, level of inoculum, and temperature of fermentation are not adequately controlled or are used irregularly. In addition to this, the recycling of yeast, which is a money saving practice extensively used in the industry and minimizes fermentation sludge, is not sufficiently practiced in BALF, creating inefficiencies.

Comparative data from leading distilleries like the National Alcohol and Liquor Factory have indicated that scientifically optimized fermentation process can reliably achieve ethanol content of 8-12% with minimal residual sugar as reported by (Beyene, 2019). The comparison itself indicates the need for scientific optimization at BALF to achieve maximum

ethanol production, more sugar recovery from molasses, and minimizing environmental pollution.

Overall, addressing these related problems is critical to the long term prosperity of Balezaf Alcohol and Liquor Factory. The factory is able to obtain more ethanol through optimizing the process of yeast propagation and fermentation and therefore less energy consumption and wastage.

1.3. Objectives

1.3.1. General Objective

The main objective of the study is to optimize yeast propagation and molasses fermentation for enhanced ethanol production while minimizing waste.

1.3.2. Specific Objectives

The specific objectives of the study are:

- ✓ To perform a thorough chemical characterization of molasses and examine the quality of process water used in ethanol fermentation.
- ✓ To investigate how varying nutrient concentrations affect yeast propagation and influence the fermentation process.
- ✓ To compare and determine ethanol content and residual sugar at optimal conditions with the baseline methods presently used and available to Balezaf Alcohol and Liquor Factory.
- ✓ To compare and assess the performance of fresh yeast with reused yeast under different propagation and fermentation conditions
- ✓ To validate the statistical soundness of the optimization models developed using Response Surface Methodology (RSM) by conducting analysis of variance (ANOVA) and model fit diagnostics.

1.4. Significance of the Study

The study will be significant since it will be able to develop solutions to major problems in ethanol production, particularly for the Balezaf Alcohol and Liquor Factory (BALF), through enhanced fermentation systems. Therefore, the significance of this study is highlighted as follows: it boosts the productivity and volume of ethanol production, reduces the production cost by virtue of the process simplification, reduces the environmental burden by virtue of the effluent formation reduction, promotes sustainable activities and renewable energy goals, and provides scalable solutions to other ethanol producers.

1.5. Scope of the Study

This study was limited to the optimization of molasses fermentation and yeast growth in order to produce ethanol at Balezaf Alcohol and Liquor Factory (BALF), Oromia Regional State, Ethiopia. This study primarily focused on optimizing the production efficiency of ethanol through conducting research on the most important technical parameters such as raw material quality, fermentation processes, nutrients quality, and the production efficiency of yeast strains. Wonji Sugar Factory process water and molasses to be employed in fermentation were chemically analyzed to determine their fermentation capacity as media.
Experimental efforts entail optimization of nutrient supplement (urea, DAP, and NPS), sugar concentration, pH, temperature, and inoculum yeast level employing Response Surface Methodology (RSM). Duplicate application of dry and recycled yeast strains at the optimum levels was done to assess their relative capability to ferment more and yield more ethanol. Economic advantage of recycling and process sustainability is also within research consideration.

Apart from this, the research examines the ecological impact of optimal fermentation by conducting lower levels of residual sugar of fermentation waste and overall efficiency improvement in the fermentation process. The results are to be utilized in giving feedback towards operational improvement and sustainable operations for BALF and can serve as a blueprint for other ethanol production plants.

Chapter Two

2. Literature Review

2.1. Fermentation Process

Fermentation is the natural process where small organisms, including yeast, ferment sugar into carbon dioxide and alcohol, particularly when oxygen is absent. Saccharomyces cerevisiae is the most common of these yeasts (Walker & Stewart, 2016). Molasses is but one of the byproducts that have been utilized extensively in the process. Molasses is a syrupy thick product that is created when sugar is being made (Anonymous, 2024). Humans create it because it's loaded with sugar and it costs nothing at all.

Molasses is primarily composed of sugars, such as fructose, glucose and sucrose. The sugars are in the range of 40% to 55% (Jamir et al., 2021; Bushu, 2014) of its composition and very fermentable. A big part of the reason why industries prefer to work with it is because it cuts out a lot of the additional steps that come with starchy compounds or cellulose. Other substances typically require pretreatment, which is time-consuming and expensive (Iwuozor et al., 2022).

Molasses seems like a good idea at the time, but it’s not sunshine and rainbows. Millions of years later, the warming and cooling of this system formed a sugar furnace of sorts, and, sugary goodness aside, there are other things in there, too a little bit of acids, salts, proteins, and even trace elements of heavy metals. These additional ingredients can do stuff to the yeast and make fermentation less effective (Beza, 2018). This is why most of the time; molasses will have to be refined before it’s usable. They are doing nothing more than diluting it or buffering its pH so that yeast has a less severe shock.

The basic process steps of molasses fermentation to produce ethanol are raw material (molasses) preparation, inoculum (yeast) propagation and final fermentation.

2.1.1. Raw Material Preparation

The success of molasses fermentation really depends on how the molasses is treated before it even gets to the fermentation stage. If you don’t handle it right, it makes it harder for the yeast to do what it needs to do. Good preparation helps make sure the yeast can get to the

sugars it needs while cutting down on the things that could mess up the process. The main things you do to prepare it are dilute it, filter it, and sometimes add extra nutrients.

2.1.1.1. Dilution of Molasses

Molasses is sticky and sweet, and on its own will actually not allow the yeast to function. What everyone will tend to do first is thin it out. This makes it so the yeast will be able to deal with it. It also reduces the concentration level of the sugar to one where fermentation will be easier. We found that experiments identified that 15% to 25% is perfect to ferment. More concentrated would kill yeast, but a lesser concentration means that your own ethanol production would be lower (Alegre et al., 2003).

2.1.1.2. Filtration

The removal of solid impurities through filtration provides the yeast with a sterile environment upon which to grow. This is important since solids remaining behind will affect yeast metabolism and efficiency of fermentation.

2.1.2. Yeast Culturing and Recycling System

The most popular microorganism to use in attaining the conversion of sugars to ethanol using fermentation is yeast. As such, yeast strain control as well as healthy yeast concentration is of vital importance in order to enable maximum yield of ethanol. A good strain should have high resistance to ethanol, hermotolerance, osmotic resistance, high fermentation kinetics, and molasses inhibitors resistance.

2.1.2.1. Yeast Propagation

After yeast propagation using molasses, the level of sugar in degrees Brix is typically lower since it gets consumed by the yeast. Fall amount depends on initial Brix, yeast strains, and propagation time. For initial molasses of 8, 10, 12, and 14 Brix, Brix fell to 4.5-8 Brix after 24 hours (Hawaz et al., 2023).

Once strain selection has been done, the yeast must be propagated to a point that it will be adequate to yield enough biomass to inoculate in the bulk fermenter. Propagation is the repeated division of the yeast in a rich nutrient medium in trying to precondition a healthy and active yeast culture for the purpose of initiating and sustaining effective fermentation

(Mije et al., 2023). Propagation generally includes: stage-wise scaling: Laboratory flasks or seed fermenters to multiple transfers into large tanks. Regulation of aeration: The oxygen is provided during initial propagation to promote growth and proliferation of yeast cells, even though it is kept low during actual fermentation so that anaerobic conditions are sustained. Maintenance of sterility: Sterilization protocols are maintained unbroken during the propagation process to avoid contamination.

The basic steps of yeast propagation are:

a. Media preparation
b. Nutrient addition
c. Inoculation

a. Media Preparation

The yeast growth medium must be able to supply all the nutrients required to sustain yeast growth at the maximum rate (Martin & Chan, 2024). Adequate preparation of propagating media lays down a health population of the yeast, whose number is proportionally related to the efficiency in fermentation.

b. Nutrient Addition

Molasses, although sweet, is devoid of other nutrients that are fundamental during the process of yeast health development, i.e., nitrogen, vitamins, and minerals. To combat such a deficiency, the same nutrients, such as ammonium salts, phosphates, or trace elements, are added to dilute molasses as countermeasures. The added nutrients catalyze yeast growth as well as provide metabolic ease to allow effective ethanol production (Beigbeder et al., 2021).

Media recipes usually involve: Carbon Sources: Besides molasses sugars, one can add other carbon sources (such as sucrose or glucose) to encourage the initial growth stages. Nitrogen Sources: Cell expansion and protein synthesis are common in cell culture with the addition of ammonium sulfate, ammonium nitrate, and urea. Trace Elements and Vitamins: Among the particular yeast cell enzymatic reactions of significance are magnesium, manganese, and zinc, which are cofactors. They are of paramount importance in yeast effectiveness, and in the case of their absence, the viability of the metabolism is affected.

c. Inoculation

Inoculation refers to inoculation of pre-inoculated yeast culture into yeast fermentative medium. This is dependent on several variables: Tolerance: Pre-adaptation of the fermentation conditions (pH, sugar level, temperature) of the yeast strain to ensure viability and function (Douradinho et al., 2023). Inoculum Rate: The cell number in the initial inoculum also controls fermentation kinetics. Large inocula levels are capable of decreasing the lag periods and enhancing the rate of alcohol formation while elevating competition for the nutrients. Pre-conditioning: Pre-acclimatization of the yeast to sublethal molasses or alcohol levels prior to fermentation will optimize its performance as well as adaptability.

Effective inoculation methods ensure rapid establishment of fermentation, improved viability of the yeast, and higher alcohol production.

2.1.2.2.1. Parameters Affecting Yeast Propagation

Yeast propagation in molasses medium is controlled by several key parameters like temperature, pH, sugar level, alcohol level, aeration, and nutrient addition. All these factors combined determine yeast growth kinetics, metabolic activity, and hence the following fermentation efficiency.

i. Temperature

Temperature directly affects yeast growth and metabolic rate (Zakhartsev et al., 2015). Optimal growth of Saccharomyces cerevisiae is at 28–32°C. Low temperatures slow down yeast metabolism, and time for propagation is longer, whereas more than 35°C may induce thermal stress, decreasing the viability of the cells and enhancing the formation of unwanted byproducts.

ii. pH

An optimal pH of 4–5.5 favors effective yeast propagation (Salihu et al., 2023). pH below 4 enhances acid stress, which inhibits yeast activity, while pH above 6 enhances the likelihood of bacterial contamination. Proper pH ensures cell membrane integrity and enzymatic processes.

iii. Sugar content

There are fermentable sugars such as sucrose, glucose, and fructose present in molasses. There has to be a desired sugar concentration to the level of 10–15 ^{0}Brix for propagation. There is osmotic stress present in yeast viability with concentrated sugar levels, but the low levels will not be able to support maximum biomass development (Bushu, 2014).

iv. Alcohol (Ethanol) Content

It is not favorable for ethanol accumulation during yeast propagation, and above 2% v/v is cytotoxic to the yeast cells as well as disrupting membrane and enzymatic functions (Walker & Stewart, 2016). Ethanol is maintained at low concentration (<1.5% v/v) by regulated levels of sugar supplement and aerobic atmospheres, promoting respiration and not fermentation.

v. Aeration

Yeast growth needs oxygen because it is important for membrane formation, especially the formation of sterol and unsaturated fatty acid. The best aeration is 0.5–1.5 vvm (volume of gas per volume of liquid per minute). Reduced aeration can lead to altered fermentative metabolism, and increased aeration leads to oxidative stress (Ukani et al., 2024).

Why is Low Alcohol in Yeast Propagation? Aerobic Conditions: Yeast cells respire instead of ferment when oxygen is present, with growth of yeast cells taking precedence over ethanol. Equilibrated Concentrations of Sugar: 10–15°Brix maintains available sugar below to be fermented as ethanol. Preference for Growth: S. cerevisiae captures sugars for yeast cell growth through respiration when oxygen is present. Expected Yield of Alcohol in Yeast Propagation Ideal range: <1.5% v/v. Maximum: >2% v/v when oxygen is limited.

vi. Addition of Nutrients (DAP, Urea, and NPS)

Yeast requires nitrogen as a macronutrient. Diammonium phosphate (DAP) and urea are the most usual nitrogen sources (Mishra et al., 2020), NPS granting nitrogen, phosphorus, and sulfur for enhanced metabolic and protein biosynthesis. In Balezaf alcohol and liquor factor DAP and urea are used as nutrients added in yeast propagation. The DAP and urea consumption in yeast propagation is 0.7-0.8 gram per liter in yeast propagation. Achieving

the ideal nutrient demand is crucial for increasing plant productivity and lowering wastewater generation.

Table 2.1: Comparison of DAP + NPS vs. DAP + Urea for Yeast Propagation:

Parameter	DAP + NPS	DAP + Urea
Sulfur Supply	Provides sulfur (for methionine, cysteine, vitamins)	Lacks sulfur; must be supplemented externally
Nitrogen Uptake	Rate Slow nitrogen release; consistent yeast growth	Increased availability of nitrogen; maintains augmented propagation
pH Control	Might suppress pH by sulfate level In an initial	pH increase (by DAP); urea hydrolysis reduces pH

Correct nutrient regulation is vital to achieve maximum cell division in yeast and to achieve the highest ethanol in continuous fermentation (van Dijk et al., 2020).

2.1.2.2. Yeast Recycling

Ethanol fermentation based on yeast recycling is a widely used industry protocol for achieving process efficiency and sustainability. Yeast recycling, which is about harnessing viable yeast cells from the waste broth and using it to make a follow-up batch. It significantly shortens and simplifies the process of new yeast isolation (Bushu, 2014). The use of the right recycling process for yeast can make ethanol producers more efficient and sustainable.

Advantage of yeast recycle is: Cost reduction: Reduces reliance on purchasing recently cultured yeast, by which lowering production costs can be achieved. Consistent Performance: Maintains yeast in a healthy and well acclimatized state from batch to batch to ensure constant fermentation performance. Reduced Lag Period: Shortens the time for the yeast to acclimatize itself to the new fermentation medium supplied, and the entire process enjoys more efficiency.

2.1.3. Fermentation Process

Ultimate fermentation refers to the action of biological transformation of fermentable sugars into ethanol by yeast. Batch or continuous fermentation is a method through which this can be done utilizing respective strengths and shortcomings.

2.1.3.1. Batch Fermentation

Batch fermentation is an exclusive system whereby constant volume molasses substrate is in the process of fermentation with addition and withdrawal possibilities or not whilst the system runs (Saini et al., 2019). The dominant characteristics are: Absolute Control: Allows greater control of variables like temperature, pH, and nutrient concentration, optimizing yeast activity. Simplicity: Easy to operate and best for small or infrequent batch runs. Restrictions: With longer fermentation time, the nutrients get used up, resulting in stationary yeast and reduced ethanol yield in the long term. Batch fermentation is common even though it is so elementary since it may not be as efficient as continuous processes.

2.1.3.2. Continuous Fermentation

In continuous fermentation, fresh substrate is continuously added to the fermenter and an equal quantity of fermented broth is withdrawn. This gives a relatively constant condition and has several benefits: Improved Productivity: Ongoing removal and addition allow for ongoing yeast activity and maximum production of ethanol (Nurhayati et al., 2014). Less Downtime: Eliminates lost time between batches such that ethanol is mass produced. Challenges: Need to be regulated very tightly so as not to rinse out the yeast and keep highest biomass concentration. Continuous fermentation, being harder to regulate, is cheaper on large scale.

Simplified Ethanol production process from fermentation of molasses in Balezaf alcohol and liquor factory is schematically shown in the fig 2.1.

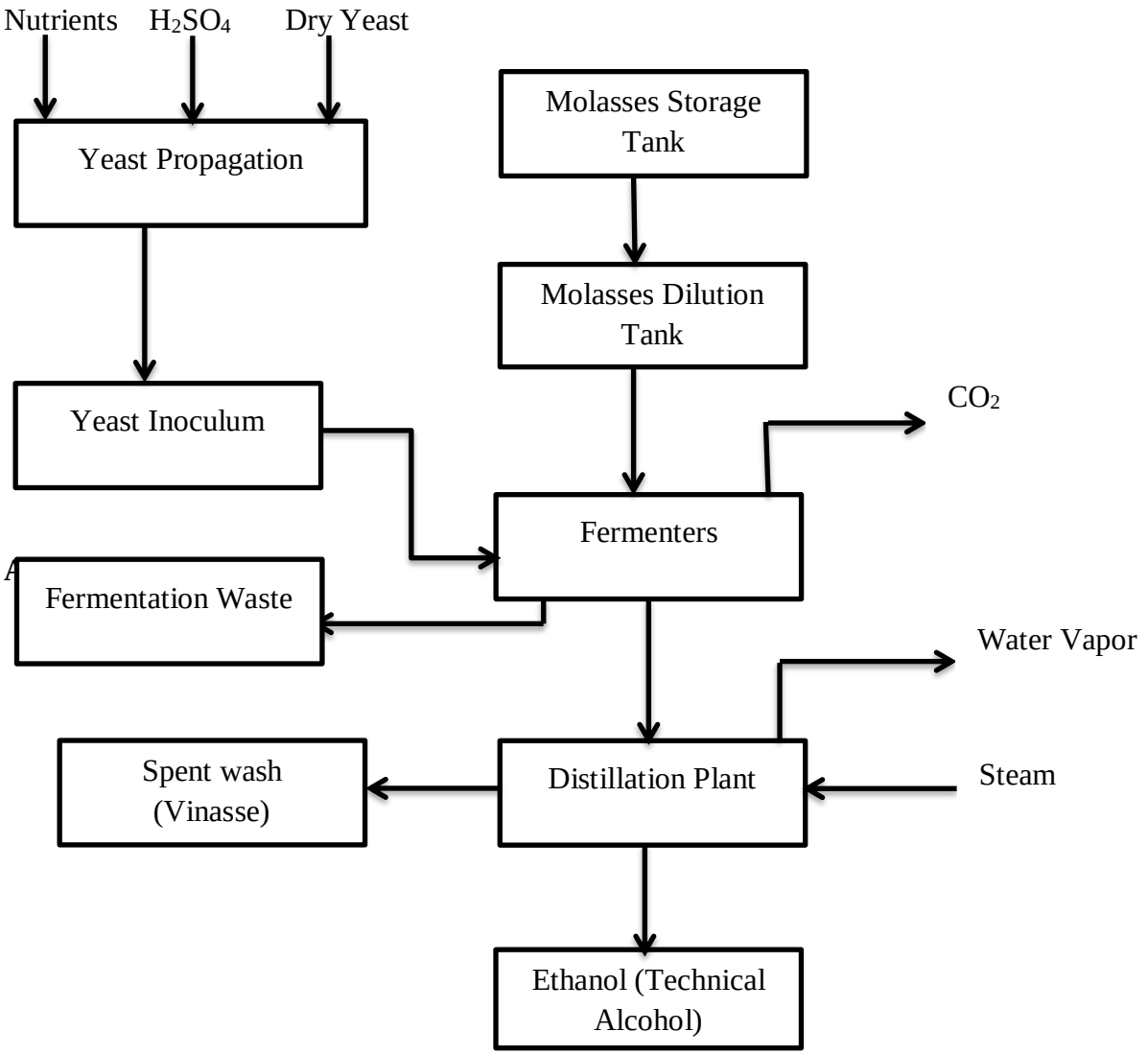

Fig 2.1: Process flow sheet of ethanol production from fermentation of molasses in BALF

2.1.4. Chemical Reaction during Fermentation

Fermentation is carried out by a chemical reaction process where glucose gets decomposed to ethanol. It is carried out in three stages, i.e., glycolysis, fermentation, and recovery of ethanol. Glycolysis degrades glucose into pyruvate. The starches get hydrolyzed by yeast enzymes into ethanol and carbon dioxide as can be observed from the reaction given below. It also generates ATP, which helps in the metabolism and growth of yeast (Yan, 2024).

Fermentation of molasses is simply the conversion of glucose into carbon dioxide and ethanol. Side reactions of fermentation will yield by-products like glycerol, organic acids, and fusel alcohols. The by-products are harmful to ethanol yield, induce unnecessary distillations, and degrade the quality of final product ethanol obtained (Tse et al., 2021).

Fusel oil synthesis, for example, is acquired through the yeast cell's amino acid metabolism during stressed fermentation (Hazelwood et al., 2008). High alcohols such as propanol and butanol fusel oils will need to be strictly controlled because they will weaken the ethanol concentration. Regulation of some processes such as fermentation's temperature level, nutrient level regulation, and genetically engineered yeast strains will decrease the formation of undesired by-products.

Molasses fermentation with these chemical reactions is:

Hydrolysis of Sucrose

$C_{12}H_{22}O11 + H_2O \longrightarrow C_6H_{12}O_6 + C_6H_{12}O$

Sucrose Water Glucose Fructose

Fermentation of glucose and fructose

$C_6H_{12}O_6 \longrightarrow 2C_2H_5OH + CO_2 + Energy$

Glucose Ethanol Carbon dioxide

It is important to know this process because there are variables that influence its effectiveness, including substrate concentration, yeast strain, and environment (Parapouli et al., 2020). For instance, glucose concentrations at high levels can be used for greater ethanol production, but because they have high concentrations, they hinder the activity of the yeast through osmotic pressure.

2.1.5. Determinants of Molasses Fermentation

There are several determinants which could potentially impact molasses fermentation efficiency significantly:

a. Sugar Concentration Influence

Evidence reveals that although increased sugar concentration may result in increased yields of ethanol, at the same time it may cause osmotic stress to yeast cells and ultimately curtail fermentation (Douradinho et al., 2023).

Original sugar concentration (°Brix) for molasses fermentation actually controls the yield and ethanol output during fermentation. It has been established through research that molasses fermentation at 25–30°Brix (14-20%) fermentable sugar can result in increased ethanol production but must be carried out under controlled conditions to prevent osmotic stress between yeast cells. Research has been established that molasses fermentation at 25°Brix can result in 4–6% (v/v) of ethanol (Raharja et al., 2019). Molasses concentrations greater than 30°Brix will inhibit yeast cell growth, leading to inefficient fermentation and higher residual sugar levels (Hawaz et al., 2024).

b. pH Effect

Metabolism of the yeast also, to a very large degree, relies on the pH of the fermentation medium. The ideal pH is 4.5-5.5, acidic to the majority of yeast cultures. Any degree of variation would result in a rate of fermentation and ethanol production being zero (Salihu et al., 2023). pH level remains constant since constant measurement and control are maintained all the time in order to allow maximum operation of the yeast.

c. Temperature Effect

Temperature also impacts performance and the yeast fermentation rate. Temperature must be 25-35°C, but temperature is expensive in terms of the higher rates of faster fermentation at the cost of weakening the yeast and reducing its performance (Salihu et al., 2023). Temperatures must be carefully measured and checked.

d. Effect of Oxygen

Oxygen needs to be supplemented in fermentation. Yeast culture will grow if the culture is kept aerobic but anaerobic to produce ethanol. Additional oxygen supply will result in the production of acetic acid and other secondary metabolites (Rosenfeld et al., 2003). Anaerobic conditions need to be supplied to provide a chance for enhancing the degree of maximum ethanol.

e. Role of Ethanol and Secondary Metabolite Inhibition

In case of ethanol accumulation, it is poisonous to yeast. Yeast metabolism is also inhibited by more than 14% v/v composition of ethanol and results in halting fermentation (Wolf et al.,

2023). Other metabolites such as organic acids and fusel oils inhibit yeast metabolism (Chidi et al., 2018). Unchecked ethanol accumulation and promotion of metabolite regulation are hence necessary.

f. Nutrient Effects in Molasses Fermentation

There are no nutrients in molasses that or beneficial to the yeast in terms of optimal performance. Nitrogen, particularly as ammonium salt and urea, is essential for protein metabolism and synthesis (Almeida et al., 2021). Phosphorus, potassium and trace minerals are also fermentation inhibitory. Addition of nutrients significantly increases fermentation rate and ethanol yield.

g. Residual Sugar

Residual sugar means unfermentable sugar after fermentation. BALF value: 2–3.5% (Balf, 2024). Ideal value: <1.5% (Abdel-Banat et al., 2010). Residual sugar over 2% would reveal defective fermentation due to yeast activity failure or due to a deficiency of nutrients and less than 1% would reveal sugars in proper usage.

h. Yeast Count

Yeast cell density at inoculation controls fermentation and antibacterial agent resistance by nature. Range: 10^6–10^8 cells/mL (Belini et al., 2017). $<10^6$ cells/mL: Slow fermentation, increased level of resistance to contamination $>10^8$ cells/mL: Foam formation, disruption of competition for the substrate. Control of level of yeast cells within its ideal range keeps fermentation under control.

i. Yeast Inoculum Size

Percentage volume inoculum of fermenter size controls the yield and lag of ethanol. Ideal range: 5–10% (v/v) (Fadel et al., 2018) < 5%: Slow fermentation, reduced ethanol yield >10%: Biomass overproduction and reduced ethanol production due to transfer of sugars to cell mass accumulation.

2.2. Distillation

Distillation is a heat separation method employed to clean and separate ethanol from the fermentation broth. Distillation is the process of heating the broth to vaporize ethanol, whose boiling point is lower than that of water, and then condensing the ethanol vapor back into liquid (Manyele, 2024).

a. Basic Distillation Techniques

The most straightforward process is single-stage distillation, where the liquid fermented and ethanol are heated and condensed and collected respectively. Although effective in preliminary separation, it cannot provide high purity ethanol because of the ethanol water azeotrope that bars it from concentrating ethanol above ~95% under normal pressure (Deepa & Nimkar, 2023).

b. Multistage and Fractional Distillation

To overcome this limitation, fractional distillation, involving the use of more than one column or distillation stage, is employed. The process provides improved separation through the use of infinitesimal differences in the boiling point of ethanol and water between stages or trays with the purpose of further concentrating ethanol (Taipabu et al., 2023).

c. Vacuum Distillation

The process by which vacuum distillation at reduced pressure reduces the boiling point of ethanol (Nassif et al., 2022). Vacuum distillation is energy conserving and avoids thermal decomposition of ethanol and thus is best appropriate in industrial manufacturing where energy economy is the primary issue.

d. Azeotropes and Entrainers

Azeotropes of the water ethanol mixture are broken and anhydrous ethanol is achieved by the method of azeotropic distillation. It involves addition of entrainers such as benzene and cyclohexane, the latter with water to create a ternary azeotrope, a broader separation (Taipabu et al., 2023).

e. Energy Considerations

Distillation is energy intensive. Some studies discuss how the most effective distillation technology vacuum, multi stage, or azeotropic can be employed in a manner to reduce heat demand and enhance ethanol yield and quality (Kooijman & Sorensen, 2022). Heat integration and energy recovery hardware can also decrease the environmental impact of ethanol production.

2.2.1. Types of columns in distillation of BALF

In Balezaf Alcohol and liquors factory the multi column distillation process consists of Degasifying column, Analyzer column, Aldehyde column, Extractive distillation column, stainless steel or stripping section column and copper or rectification column. The distillation section strips off the ethanol from 6.5-9.5% fermented wine and increases the concentration of ethanol up to 96.7%.

Initially, Fermented wine with alcoholic grade 6.5-9.5% v/v is pumped to decanter from each fermentation tank. The decanter separates the fermented alcohol from the sediment. In this case the sediment is settled at the bottom and the fermented wine is pumped to degasifying column after slow increasing the flow rate of fermented wine up to 10 m^3/h and preheating the wine by spent wash water (from analyzer column) through the two plate heat exchangers working interchangeably.

a. Degasifying column

Degasification is the removal of unwanted dissolved gases from the liquid. The main aim is removing dissolved gases in the wine in degasifying column. The heated wine with temperature of 60-70^{o}c enters to degasifying column tangentially. So the dissolved gasses are separated by that temperature make them to vent up. Phase boundary is created that means liquid and gas separation happen. The vent gasses are leaves at top of the column and passes to plate type heat exchanger. The heat exchanger cools the vent gasses using cold water from cooling tower. After cooling the water goes to cooling tower. The output of the heat exchanger is degasing head 1 (acetaldehyde) collected as 5lit/hr and the non-condensing gas leaves as degasing vent. The bottom product is degasified wine leaves from the bottom of the column and then to analyzing column.

b. Analyzing column

It is a tray type distillation column which contains sieve tray with two down comer in one side. It has 2.4 plate thickness and 505 cm radius. The operating pressure and temperature is 150-350 mbar and 86-90.5^{o}c respectively. So, this tray type column is selected due to how

reduce the scale because if we used packed type the effect would larger than tray type. The scale is come from the fermented wine from fermentation due to the addition of sulpheric acid and lack of molasses purification plant in the company. Due to this $CaSO_4$ has probability formed when heating the wine by steam in the analyzer.

The degasified wine is fed to this column from side and then moves down through down comers. The steam is enters to through the base of the column and rises up through the opening of the tray. As the steam passes through the tray, heart transfers to the wine then the liquid start to boil. The continuous contact between steam and liquid results for separation of alcoholic and non-alcoholic components. The alcoholic component has low boiling point that means it can boil easily and quickly. The non-alcoholic component has high boiling point not boil by the given heat, they call spent wash some alcohol present with it. The spent wash leaves the column at base of the column and sends to 6m underground for returning the alcoholic contents with steam to lower side of analyzing column. The steam free spent wash returned with narrower tube with increased pressure to preheat the fermented wine before enter to degasifying column.

The vapour leaves through the top of the column is sent to aldehyde for further purification.

c. **Aldehyde column**

It is packed bed type column and used to separate more volatile components from less volatile components. It's operating pressure and temperature is 74-76^{o}c and 100mbar respectively. It works using volatility difference.

The vapor that leaves the analyzer column enters to lower side of the aldehyde column. The more volatile components vent up as top product and less volatile components as lower product. The top product pass to two heat exchangers and then it condenses. The vapor that does not condense is first condenses to next heat exchanger. From the condensing vapor in the both heat exchanger 100lit/hr is leaves and the remaining return back to the column as reflux. The bottom product is raw alcohol or katikala which has alcohol grade of 35-50%v/v. It passes to heat exchanger to be cooled and then store in the shift tanker.

d. **Extractive distillation column(ED)**

Extractive distillation is used for separation and uses a separation of solvent which has high boiling point and miscible with the mixture, but that does not form an azeotropic mixture. The solvent interact with components of the mixture result to change the relative volatility. It is packed type column with operating temperature and pressure of 132-137^{o}c and 2.8-3 bars. The raw alcohol from shift tanker pumped to the re-boiler is preheated by its output alcohol (8% alcohol) which is heated by steam so heat transfer from 8% alcohol to raw alcohol. This

heated raw alcohol is fed to ED through flow meter. The water from stainless column is also heated in the re-boiler and then enters above the raw alcohol fed position for showering. The steam comes from the re-boiler enters to lower side of column. It is used to separate from more volatile from no- volatile by that temperature. The vapor that has great volatility with temperature of 132-137^{o}c leaves as top product. This vapor condenses using water (70^{o}c) came from the stainless column after that the water return back to stainless steel column. Head 3 is collected 100lit/hr from condenser then it pass through heat exchanger for cooling stored in the tanker and the rest reflux back to top side of column.

The bottom product with solvent has 8-10%v/v diluted alcohol passes to re-boiler to heat the water and the raw alcohol. Then the diluted alcohol enters into stainless steel.

c. Stripping section (stainless steel column)

It is packed type column with operating pressure and temperature of 250-450mbar and 75-81^{o}c respectively. The main aim of this column is to remove much of the water and separate fusible and light oil from the alcohol mixture. The diluting alcohol from ED enters medium side and the steam enters at base of stripping section. Since alcohol is more volatile than water this feed steam makes the alcohol to rises up and the lease water leaves at bottom of the column. The lease water send to 6m underground to trap some vapors that contain alcohol and it passes to plate heat exchanger to preheat the boiler feed water. Light oil (ethyl acetate) and fusel oil (amyl alcohol) extracted near top side of the column and collected each 40lit/hr with alcohol grade of 70%v/v.

d. Copper column (rectification column)

This column operates under a temperature of 72-74^{0}c and a pressure of 250-450mbar to upgrade the concentration of ethanol also separate methanol from mixture due to higher affinity of copper towards methanol, the copper makes methanol to stay in the column rather than leaving with ethanol. In this column the vapor leaves at top and passes to main condenser and that couldn't condense pass to vent condenser to prevent vapor loss. The vapor that couldn't condense in the two condensers is released to atmosphere. 40lit/hr head 2 main and 40lit/hr head 2 vent is collected pass to denature tank after cooled by heat exchanger and the remain make reflux. The condensate with low grade of alcohol pumped from lower side of this column to upper side of stripping column to strength its alcoholic content. The product leaving this column i.e pure alcohol has 96.8%v/v alcohol grade is cooled and collected in the pure alcohol storage tank.

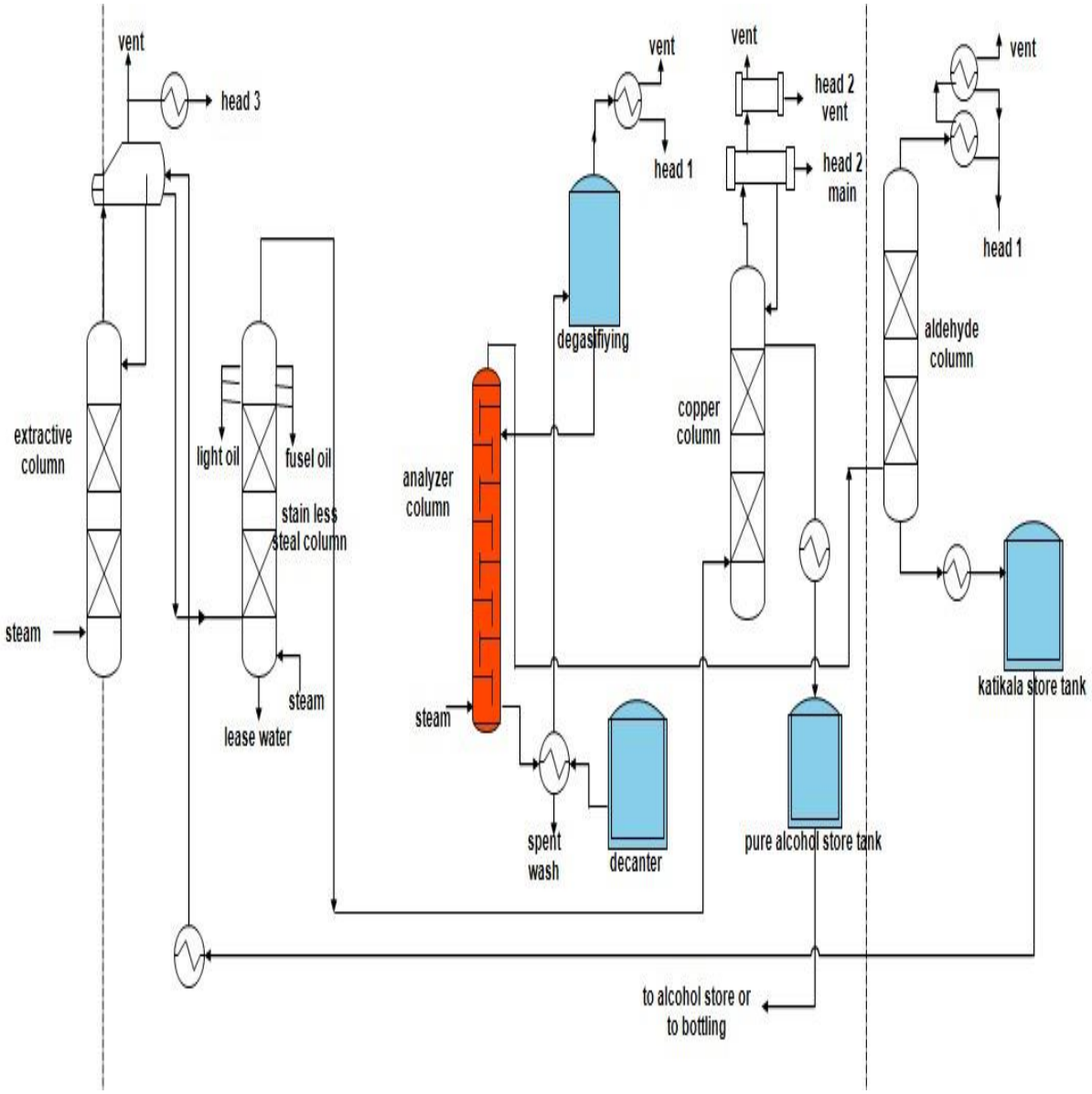

Fig 2.2: Process flow diagram of distillery plant in BALF

2.3. Fermentation Sludge and Distillation Wastewater Treatment

Fermentation waste of molasses, or sludge, is byproducts from ethanol production with residual sugars, microbial biomass, and other organics. High in organic materials, it can be both an environmental degradant if wasted and a resource for biogas or making compost (Mikucka & Zielińska, 2020).

Treatment of vinasse or spent wash, as it's popularly called, is a major environmental part of ethanol processing. Vinasse is produced on a huge scale from the fermentation and distillation of molasses and contains high organic matter in the form of residual sugars, yeast

cells, acids, and minerals. Raw vinasse, if released into water bodies and soil, may contaminate water and land by virtue of its high BOD and COD.

2.3.1. BOD and COD characterization

Massive amounts of spent wash (effluent) are produced in ethanol manufacturing in the process of molasses fermentation. The effluent contains high BOD and COD values since it contains organic contents, residual sugars, volatile acids, and other byproducts of fermentation. Proper analysis of such parameters is required in pollution load estimation and planning effective wastewater treatment plants (Satyawali & Balakrishnan, 2008).

Currently, the BOD and COD value of molasses fermented waste at Balezaf Alcohol and liquor factory (BALF) is 60,000-90,000 mg/L and 100,000-150,000 mg/L respectively. From the fermented molasses wine 10-15% (from 1 L of fermented wine 0.1-0.15 L) is decanted and stored in a storage tank at the bottom and disposed as sludge or fermentation waste at the end of the process (Balf, 2024).

Chapter Three

3. Materials and Methods

3.1. Materials and Reagents

a. Raw Materials

Materials utilized throughout the course of this research were molasses from Wonji Sugar Factory, which is used as the primary substrate for fermentation, and process water used in dilution of all sample preparations. DAP (diammonium phosphate), urea and NPS fertilizer were utilized where necessary nutrient additives to facilitate yeast growth and efficiency in fermentation. Recycled and dehydrated yeast cultures were utilized for propagation and fermentation trials in controlled laboratory settings.

b. Reagents

These reagent analysis chemicals applied for chemical tests and quality analysis included sulfuric acid (H_2SO_4), hydrochloric acid (HCl), sodium hydroxide (NaOH), phenolphthalein indicator, Fehling's solution, EDTA, 10% w/v lead acetate solution, sodium phosphate-potassium oxalate solution, ammonia solution, ferrocyanide, methylene blue, sodium thiosulfate, liquid paraffin, triethanolamine, sodium sulfide, Patton and Reeder's indicator, ferrous ammonium sulfate, and potassium dichromate. These reagents were applied for titration, complexometric titration, sugar analysis, and nitrogen estimation in the molasses and the fermentation medium.

c. Equipment

The laboratory procedures used different sets of equipment to make the experimentation process easier. They consisted of beakers, Erlenmeyer flasks, volumetric flasks, glass rods, burettes, pipettes, thermostatic water baths, analytical balances, muffle furnaces, heating equipment, incubators, refractometers, pH meters, hemocytometer equipped microscopes, fermenter vessels, filters, centrifuges, mixers, ebulliometers, air blowers, and thermometers. The equipment helped in precise measurement, sample manipulation, and fermentation control during the study.

3.2. Description of the Sampling Area

Balezaf Alcohol and Liquor Factory (BALF) is situated in the Oromia Regional State of Sheger City, 20 kilometers southwest of Addis Ababa. It currently has three branches: the head office, the Sebeta Town production unit, and the Wonji Shoa ethanol plant, which is under construction. The factory's distillery plant generates an average capacity of producing 6,400 m^3 of alcohol annually and 42,840 m^3 of distillery spent wash annually (Balf, 2024). The factory has 500 permanent staff and 120 seasonal staff.

3.3. Methods

3.3.1. Characterization of Input Materials

Molasses and process water characterization was done to understand their fermentation potential along with the factors affecting yield of ethanol. Composition analysis of molasses was conducted in terms of fermentable sugar content, unfermentable sugar, brix, calcium content, ash content and volatile acid level in molasses. Input water quality analysis is done in terms of residual chlorine, pH and hardness as well.

3.3.1.1. Compositional Analysis of Molasses

I. Calculation of Total Reducing Sugar

Reducing sugar was quantitated by dissolving molasses to the extent of 1 g in distilled water in 100 ml volumetric flask. 50 ml solution was taken in a beaker and to that; 5 ml of hydrochloric acid (HCl) was added. It was kept at 65°C for 20 minutes using a water bath to hydrolyze the sugar. The solution was rapidly cooled above, neutralized with NaOH, acidified with 0.5 M HCl and brought to 100 ml. The titration was performed by heating Fehling's solution A and Fehling's solution B with 10 ml sugar solution and 30 ml distilled water in a conical flask. Sugar solution in the boiling state was added drop wise and an indicator, methylene blue, was utilized. Titration was done if no blue color and red cuprous oxide precipitate had been obtained, which would complete the process (Mangwanda et al., 2023)

$$\textbf{Total Reducing Sugars} = \frac{\text{Fehling Factor (F)} \times 0.103 \times 100 \times 100}{\text{Titration Volume (V) in ml}}$$

II. Determination of Unfermentable Sugar

Unfermentable sugar was determined by Praj standard method (Thiers et al., 1948). It was isolated by fermenting 2 g molasses with 20 g compressed fresh yeast under incubation at

30°C for 24 hours. Fermentation sample was filtered out to exclude yeast and other solids. Filtrate was deled and led under deleading for removal of interfering material before titration with Fehling's solution as per total reducing sugar determination. The remaining sugar left over from fermentation was then determined in an effort to obtain the unfermentable sugar.

$$\textbf{Unfermentable Sugars (\%)} = \frac{5.127}{\text{Titer (v)} \times \text{Fehling factor (F)} \times \text{Dilution factor (D)}}$$

III. Analysis of Fermentable sugar

Fermentable sugar content was determined by subtracting total unfermentable sugar content from total reducing sugar content (Thiers et al., 1948). This is the fermentable sugar content to be fermented, which is important in alcohol production.

Fermentable Sugars (%) = Total Reducing Sugar – unfermentable Sugar

IV. Sulphated Ash Analysis

Ash was determined by ICUMSA conductivity methods (Mckee et al., 2013). Three grams of molasses were placed in a red hot crucible in which the quantity of sulphated ash needed was to be determined. The sample was slowly carbonized by using moderate heat so that organic material was incinerated and a few pure drops of sulfuric acid (H_2SO_4) were added to carry out the decomposition. Subsequently, the crucible was subjected to heating at 550°C for initial ashing and further up to 800°C for complete combustion to constant weight in a muffle furnace. Percentage weight of residue of sulphated ash of original sample weight was the residue remaining in the crucible.

$$\textbf{Sulphated Ash (\%)} = \frac{100 \times \text{mass of the residue and crucible(M2)} - \text{mass of empty crucible (M0)}}{\text{mass of the original sample (M1)}}$$

V. Volatile acidity analysis

Steam distillation was utilized to estimate volatiles acidity (Alaimo, 2025). Molasses 50 g was taken and mixed with 250 ml of distilled water and acidified with 1 ml concentrated sulfuric acid to remove volatile acids. It was distilled and 100 ml of distillate was harvested. Distillate was titrated with 0.1 N NaOH with 2 drops of phenolphthalein as an indicator. Abrupt pink color and short lived acidity showed the endpoint in parts per million (ppm) acetic acid.

$$\textbf{Volatile acidity (ppm)} = \frac{\text{Titer sample} \times \text{normality solution of NaOH } (0.1) \times 60000}{\text{recovery factory of the distillatio assembly } (0.65) \times \text{sample taken } (50 \text{ ml})}$$

VI. Analysis of Calcium as (CaO)

Calcium was determined by EDTA complexometric titration (Kimaru et al., 2018). 15 gram of feedstock was transferred to 250 ml volumetric flask with distilled water. 2.5 ml from the diluted sample was taken and 100 ml of distilled water is added. 10 mg sodium sulfide and 2-3 drops of triethanolamine were employed as interference eliminator reagents after the solution of a dilute sample. An appropriate buffer solution was employed for the increase of the pH of the mixture to 12.5. Titration was performed after the addition of Patton and Reeder's indicator with EDTA solution to a point where the endpoint was marked by a color change from violet red to blue green. The amount of EDTA employed was subsequently applied to calculate the level of calcium oxide (CaO).

$$\textbf{Calcium (in gm)/100 gm molasses (\%w/w)} = \frac{\text{Titer reading (ml)} \times 1.002 \times 100}{0.15 \times 1000}$$

VII. Brix Measurement with Refractometer

Percentage of soluble solids in the sample, i.e., Brix, was determined using a refractometer (Mangwanda et al., 2023). Refractometer prism was specked with a small amount of molasses solution and immersed in the balance case at a temperature of 20°C. Brix on the refractometer scale reading was directly obtained.

Brix of Molasses = 65–85°Brix

VIII. Dry Solids Test (Test for Total Solids)

5 g molasses was accurately weighed into a pre-tared and pre-dried plate for the analysis of total solids. The sample was oven dried at 105°C for 3–4 hours to constant weight. Total solids were expressed as percentage final dry weight to original sample weight (Mangwanda et al., 2023)

$$\textbf{Total Solids (\%)} = \frac{\text{Weight of dish plus dried sample} - \text{Weight of pre-dried dish}}{(\text{Weight of dish plus molasses} - \text{Weight of pre-dried dish}} \times 100$$

IX. Analysis of Nitrogen and Analysis of Protein

Nitrogen was calculated by Kjeldahl process (Maehre et al., 2018). 1.0 g molasses sample was digested in a definite volume of concentrated sulfuric acid (H_2SO_4) having copper

catalyst at 400°C until the mixture was clear. Solution was neutralized by NaOH and distilled and ammonia evolved was absorbed in boric acid. Trapped ammonia was titrated to endpoint with 0.1N HCl. Nitrogen was measured on an absolute basis and protein content computed from the nitrogen reading by multiplying 6.25.

$$\textbf{Total Nitrogen (\%)} = \frac{\text{volume of HCl used for titration} - \text{volume of blank titratio} \times \text{normality of HCl} \times 1.4}{\text{sample weight (g)}}$$

Protein (%) = Total Nitrogen (%) × 6.25

X. Polarization Analysis

A polarimeter was applied during the polarization test (Vela, 2021). The elimination of molasses from the sample was carried out through dissolving the solution in a lead acetate solution. Filtered lead acetate solution was filled to the mark in a polarimeter tube after solution filtration. The measured angle of polarization (°Pl) was utilized in the calculation of sucrose content.

$$\textbf{Polarization (\%)} = \frac{\text{Observed Polarization}}{\text{Standard Polarization}} \times 100$$

Table 3.1: Analysis of Molasses

S.N	Type of analysis	Unit	Standard range	Result
1	Total reducing sugar	%	40-60	*
2	Unfermentable sugar	%	2-5	*
3	Fermentable sugar	%	30-50	*
4	Brix	Degree Brix	65-85	*
5	Nitrogen	%	0.1-0.5	*
6	Sulphated Ash	%	5-15	*
7	Calcium as (CaO)	Ppm	1500-2000	*
8	Dry solids	%	75-85	*
9	Volatile acidity	Ppm	2000-5000	*
10	Protein	%	2-5	*
11	Pol	%	28-30	*

Source: - Food and Agricultural Organization (FAO) publications on sugar and molasses; Journal of Agricultural and Food Chemistry (AFC) articles discussing the chemical composition of molasses.

3.3.1.2. Physicochemical Analysis of Process Water

I. Total Dissolved Solids (TDS) Analysis

A representative sample (typically 100 mL) of filtered sample water is passed quantitatively to a clean pre-tared evaporating dish. The sample is dried in an oven to constant weight at 180°C for at least one hour or to constant weight. The dish is dried, allowed to cool to room temperature, and weighed (Adjovu et al., 2023).

$$\textbf{TDS (mg/L)} = \frac{\text{Weight of dish with dried residue (mg)} - \text{Weight of clean,empty dish (mg)} \times 1000}{\text{Volume of sample taken (ml)}}$$

II. Hardness Analysis

Water hardness was determined by EDTA titration (Dhoke, 2024). pH of the water sample was adjusted to 10 with the help of ammonium chloride buffer. Ammonium chloride buffer solution was added and then Eriochrome Black T indicator, which indicated wine-red color in presence of magnesium and calcium ions. The water sample was titrated with 0.01 M EDTA until a transition of wine-red color to pure blue was achieved, which was taken as end point. Water hardness was quoted in terms of $CaCO_3$ equivalent.

$$\textbf{Hardness as mg } \textbf{CaCO}_3\textbf{/L} = \frac{\text{ml of titrant for sample} \times \text{mg of CACO3 equivalent to 1.0 ml EDTA titrant} \times 1000}{\text{ml of sample taken}}$$

III. Chlorine Analysis

Chloride was ascertained by argentometric titration (Farooq et al., 2023). Sulfuric acid was added to the sample until pH 7–10 to acidify the sample. Potassium chromate indicator was added and became yellow color. Known strength solution of silver nitrate ($AgNO_3$) was added to the sample until the color became pinkish yellow, which was the end point. The chloride concentration was calculated using the quantity of silver nitrate used.

$$\textbf{Chlorides (mg/L)} = \frac{(\text{ml of titration for sample} - \text{ml of titration for black}) \times \text{normality of AgNO3} \times 35450}{\text{ml of water sample taken}}$$

Table 3.2: Analysis of Process Water

S.N	Parameter	Unit	Standard Range	Result
1	TDS	mg/L	< 500	*
2	pH	-	6.5-8.5	*
3	Hardness	mg/L $CaCO_3$	0-200	*
4	Chlorine	mg/L	0.2-4.0	*

Source: - WHO: "guidelines for drinking water quality"; American Water Works Association (AWWA): "a hand book on drinking water".

3.3.2. Optimization of Nutrient and Sugar Concentration

Response Surface Methodology (RSM) is applied to investigate the influence of changing sugar and nutrient content on yeast culture growth and the fermentation process. The initial advantage of RSM is that it requires fewer experimental runs to produce statistically significant data (Beigbeder et al., 2021). Box-Behnken Design was employed to explore the interaction between various sets of nutrients and produce response surface plots and ANOVA summaries due to its efficiency compared to other designs of the response surface, e.g., Central Composite Design, when it comes to performing three variables at three levels. It also circumvents attempting extreme experimental conditions, which prevents experimental failure or unrepresentative data points, particularly in biological systems like yeast fermentation.

3.3.2.1. Experimental Design for Yeast Propagation

Optimization of yeast propagation process was affected by three independent variables: pH, fresh yeast dose, and nutrients (DAP, urea, and NPS) each at three levels. The nutrient concentration is 75% of DAP and 25% of urea or NPS. Response variable is yeast cell count in yeast propagation. The generation of alcohol is not advised in yeast propagation because the aim of yeast propagation is to prepare a strong, healthy and large yeast population that can ferment sugar efficiently in to ethanol in fermentation process.

Table 3.3: Experimental Design for Yeast Propagation

Run	Independent factors	Level	Amount	Unit
1	pH	3	4.0, 4.75 & 5.5	
2	Nutrients (DAP and urea or NPS)	3	0.5, 1.0 & 1.5	g/L
3	Fresh yeast dose	3	1.00, 1.50 & 2.00	g/L

3.3.2.2. Yeast Propagation Experiment Trial

Yeast propagation was carried out in 10 liter fermenters in diluted molasses of 8.34% concentration. Nutrients (DAP and urea/NPS) and fresh yeast were added as per the Box-Behnken Design matrix. The blend was kept at room temperature and stirred continuously. Aeration was provided at a flow rate of 1.0 vvm for the first 17 hours to induce aerobic growth of yeast. The samples were taken to measure sugar uptake (°Brix) and yeast cell that was counted in a hemocytometer and viewed microscopically.

Table 3.4: Experimental set up for fresh yeast and other nutrients (DAP and urea)

		Factor 1	Factor 2	Factor 3	Response
Std	Run	A:pH	B:Nutrients (DAP+urea)	C:Fresh Yeast	Yeast cell count
			g/L	g/L	cells/ml
5	1	4	0.5	1.5	*
1	2	4.75	1	1.5	*
17	3	4.75	1	1.5	*
14	4	5.5	1	2	*
12	5	4.75	1	1.5	*
4	6	4	1	2	*
6	7	4.75	1	1.5	*
13	8	4	1	1	*
8	9	5.5	1.5	1.5	*
9	10	4	1.5	1.5	*
3	11	4.75	0.5	2	*
2	12	4.75	1	1.5	*
16	13	4.75	0.5	1	*
11	14	5.5	0.5	1.5	*
7	15	4.75	1.5	1	*
15	16	4.75	1.5	2	*
10	17	5.5	1	1	*

3.3.2.3. Experimental Design for Fermentation

Optimized factors are sugar concentration, inoculum grown yeast, and fermentation temperature. All three factors were set at three levels. The response factors are alcohol content in fermented wine and residual sugar content in fermented wine upon completion of fermentation.

Table 3.5: Experimental Design for Fermentation

Run	Independent factors	Level	Amount	Unit
1	Sugar concentration	3	14, 17 & 20	%
2	Temperature	3	25, 30 & 35	^{0}C
3	Yeast inoculum	3	5, 10 & 15	%

3.3.2.4. Experimental Setup for Fermenting Dry Yeast Inoculum

1 and 5 L fermenters were utilized in RSM analysis. Sugar concentration, size of yeast inoculum, and temperature were controlled. Experimental values of optimum sugar concentration, yeast inoculum, and fermentation temperature were used in experiments. All the experiments were tested for their effects on total ethanol production and rates of fermentation. Molasses blend was fermented for 48 hours.

3.3.2.5. Analysis of Alcohol Content, Residual Sugar Concentration, and Yeast Cell Mass in Yeast Propagation and Fermented Wine

The development of the yeast was promoted by monitoring following sugar content (Brix) and yeast cell count at an interval of six hours in yeast propagation. The fermentation process was checked through the measurement of residual sugar and ethanol content in wine.

a. Calculation of Alcohol%

The alcohol content in fermented wine is determined by using ebulliometeric method (Susial-Badajoz et al., 2023). The ebulliometer provides alcoholic strength of fermented wash as boiling point directly, which decreases with rise in alcohol content. Calibration begins where it does: the chamber of the ebulliometer is filled to level with distilled water, placed on boil, and brought to equilibrium. Boiling point of distilled water (approximately 100.0 °C at ordinary atmospheric pressure) is noted down and the setting of calibration adjusted correspondingly.

Second, fermented wash sample is initially prepared by degassing from dissolved CO_2, suspended solids, and yeast cells. Slightly stir the sample in an effort to degas and expel dissolved CO_2, which would affect boiling point. Measurement involves draining water from ebulliometer and filling same with sample of prepared fermented wash. Heat sample slowly to boiling point and stabilized boiling point is measured. Finally, percentage alcohol can be derived from a measured boiling point with a reading of alcohol concentration conversion chart provided with the ebulliometer to indicate percentage alcohol (v/v) ethanol.

Ethanol % (v/v) = Reading taken off conversion chart against boiling point

b. Calculation of percentage residual sugar

For residual sugar analysis, 10 mL of the fermented wash is taken and diluted to volume in a 100 mL volumetric flask using distilled water. The mixture is shaken well, and the wash diluted is filled in a 50 mL burette. For titration, 5 mL of B solution and 5 mL of A solution of Fehling's are taken into a 500 mL Erlenmeyer flask and 30 mL of distilled water and 15 mL of diluted fermented wash from the burette are poured into it. The flask is then set over a hot plate and boiled gently until a bright brick-red coloration is observed. 4 to 5 drops of methylene blue indicator are added here. Titration proceeds under gentle boiling conditions by adding small volumes of the diluted fermented wash from the burette in steps (about 2–3 mL at a time). Towards the endpoint, diluted wash is dropped slowly to create the desired effect. The endpoint is calculated from a color shift from blue to red brick with precipitate of red. The volume taken is noted and the remaining sugar by usual Fehling's titration equation (Hemamalini et al., 2019).

$$\textbf{Residual Sugar (\%)} = \frac{5.127}{\text{Burette Reading} \times \text{Fehling's Factor} \times \text{Dilution Factor}} \times 100$$

c. Analysis Yeast Cell Count (Cells/ml)

The cell count of the yeast in the fermented wine is counted by hemocytometer microscopy (Provost & Wallert, 2015). The sample is pre-dilution previously in advance in sterile distilled water, typically by a ratio of 1:100 for counting. Optional methylene blue staining may be used to differentiate between dead and living cells where dead cells stain blue while living cells do not change their color. The hemocytometer is then filled with a clean cover slip to use as a cover over it and a pipette holding 10 µl of diluted sample placed in the

chamber. The sample takes 1–2 minutes prior to examination on a microscope. At 40X, five of the large squares of the hemocytometer grid are counted for counting yeast cells. The cell number per square is averaged and applied to the standard hemocytometer formula to give the number of total yeast cells per milliliter (cells/ml).

$$\textbf{Yeast cells (Cells/ml)} = \frac{\text{Average Count per quare} \times \text{Dilution Factor}}{\text{Volume of Square (0.0001 ml)}}$$

3.3.3. Effect of Yeast Recycling on Yeast Propagation and Fermentation Process

Recycled yeast is obtained from previous fermentation, while fresh yeast is obtained from commercial stocks. Both are tested for viability and activity prior to use.

Yeast recycling is preceded by recovery of fermented broth from molasses fermentation tank to manageable level, hence minimizing contamination. The broth is then transferred into sterile vessels (Erlenmeyer flasks) for treatment. Centrifugation of the harvested broth is done at 3000 rpm for 10 minutes in an effort to centrifuge yeast cells from the harvested broth and ultimately slow down decantation of the supernatant without any disruption of the yeast cell pellet. Pellet washing is performed using sterile phosphate buffered saline (PBS) and centrifuged at 3000 rpm for 5 minutes to wash the yeast. This is repeated 2 to 3 times in an effort to remove the excess of the products of fermentation. Later, yeast viability is established by the hemocytometer and microscope (Revvity, 2024).

3.3.3.1. Experimental Set up for Recycled Yeast Propagation

All processes in this section are the similar to experimental set up prepared in table 3.4. The only difference is that the fresh yeast is replaced by recycled yeast for the purpose of comparison in Yeast propagation and fermentation process.

3.3.3.2. Experimental Design for Recycled Yeast Inoculum Fermentation

Parallel fermenters were carried out under the initiation of fresh and recycled yeast inoculums. Same is employed in all fermentations for the purpose of comparison. Yeast viability, residual sugar, and ethanol yield are examined. To determine the impact of recycling on efficiency of fermentation, the data is statistically compared.

Chapter Four

4. Result and Discussion

4.1. Analysis of Molasses and Process Water

Composition analysis of molasses is conducted in terms of fermentable sugar content, unfermentable sugar, brix, calcium content, ash content and volatile acid level in molasses. Input water quality analysis is done in terms of residual chlorine, pH and hardness as well.

4.1.1. Compositional Analysis of Molasses

Compositional analysis of molasses for sugar content revealed total reducing sugar content to be 49.52%, falling within the range (40–60%) most suitable for optimum production of ethanol. Fermentable sugar was 46.02%, reflecting good availability of substrate in the form of sucrose, glucose, and fructose available for yeast utilization and supportive of optimum fermentation. Unfermentable sugar level was 3.5%, which is in a healthy range of 2–5%. Less unfermentable sugar is preferred because it prevents wastage and maximizes use of sugar as ethanol (Bushu, 2014).

Brix reading was 80°, which is much dissolved solids concentration. While it assures high concentration of sugar, it also demands proper dilution before fermentation. High Brix values bring into play the effect of causing osmotic stress in yeast and preventing propagation and fermentation. Ideal Brix adjustment to around 13–15° during yeast propagation was thus necessary in this research. Nitrogen content was 0.14%, close to the lower limit of the normal range of 0.1–0.5%. Since molasses lacks nitrogen, necessary for yeast cell development, this accounts for why the use of DAP, urea, or NPS is needed. Sulphated ash content was 16.55%, which exceeded the specification upper limit of 15% (Lola et al., 2023). Excess ash content is an issue as it would mean that there are inorganic impurities present that would inhibit yeast activity and lead to increased scaling of the distillation columns. This says a lot about the need for more molasses clarification before fermentation.

Calcium in the form of CaO was 2250 ppm, more than the wanted maximum of 1500–2000 ppm. High calcium will lead to scaling in heating and distillation equipment, and induces salt stress inhibition of fermentation. Volatile acidity was 4820 ppm, at the higher safe limit. Volatile acids (mainly acetic acid) at too high a level inhibit yeast growth, and hence

molasses conditioning or neutralization prior to preparation. 3.10% protein content and 29.51% polarization (Pol) value were in normal range and indicate the availability of amino acids and residual sucrose beneficial to yeast nutrition and fermentation (Bushu, 2014).

Table 4.1: Analysis of Molasses

S.N	Type of analysis	Unit	Standard range	Result
1	Total reducing sugar	%	40-60	49.52
2	Unfermentable sugar	%	2-5	3.50
3	Fermentable sugar	%	30-50	46.02
4	Brix	Degree Brix	65-85	80.00
5	Nitrogen	%	0.1-0.5	0.14
6	Sulphated Ash	%	5-15	16.55
7	Calcium as (CaO)	Ppm	1500-2000	2250
8	Dry solids	%	75-85	81.50
9	Volatile acidity	Ppm	2000-5000	4820
10	Protein	%	2-5	3.10
11	Pol	%	28-30	29.51

4.1.2. Physicochemical Analysis of Process Water

Process water analysis indicates that all parameters examined are normal. 250 mg/L TDS, which is far below the suggested 500 mg/L, is a sign of moderate mineral concentration. The pH level of 7.2 is between neutral and best for most industrial operations. Water hardness is extremely low at 15 mg/L (as $CaCO_3$) to avoid scaling and for long equipment life. Chlorine levels are 2.5 mg/L, in the range that is safe for disinfection, but it must be removed if the water is to be consumed for fermentation purposes in order not to hinder microbial growth (Muhammad et al., 2014). The water quality avoided potential inhibition of fermentation or precipitation of nutrients.

Table 4.2: Analysis of Process Water

S.N	Parameter	Unit	Standard Range	Result
1	TDS	mg/L	< 500	250
2	pH	-	6.5-8.5	7.2
3	Hardness	mg/L $CaCO_3$	0-200	15
4	Chlorine	mg/L	0.2-4.0	2.5

4.2. Optimization of Nutrients, Sugar Concentration and Other Factors in Yeast Propagation and Fermentation Process

Molasses with brix of 14 degree brix was prepared for yeast propagation in 10 L capacity. Nutrients (DAP and urea or NPS) and fresh yeast are added in prepared solution with controlled pH and at room temperature. The yeast propagation was conducted for 17 hours. The fermentation is conducted at a sugar concentration of 14%, 17%, and 20%, a temperature of 25°C, 30°C, and 35°C, and a yeast inoculum size of 5%, 10%, and 15%. The following data were generated by using Box-Behnken Design. The design program suggested a quadratic model for this response to test its adequacy and describe its variation with independent variables.

4.2.1. Optimization of pH, Nutrients (DAP and Urea) and Fresh Yeast in Propagation

Table 4.3: Optimization for pH, Nutrients (DAP and Urea) and Fresh Yeast Dose Results in Yeast Propagation

		Factor 1	Factor 2	Factor 3	Response
Std	Run	A:pH	B:Nutrients (DAP+urea)	C:Fresh Yeast	Yeast Cell Count
			g/L	g/L	cells/ml
5	1	4	0.5	1.5	2.68E+08
1	2	4.75	1	1.5	3.48E+08
17	3	4.75	1	1.5	3.43E+08
14	4	5.5	1	2	3.08E+08
12	5	4.75	1	1.5	3.44E+08
4	6	4	1	2	2.72E+08

6	7	4.75	1	1.5	3.5E+08
13	8	4	1	1	2.67E+08
8	9	5.5	1.5	1.5	2.7E+08
9	10	4	1.5	1.5	2.53E+08
3	11	4.75	0.5	2	3.06E+08
2	12	4.75	1	1.5	3.52E+08
16	13	4.75	0.5	1	2.78E+08
11	14	5.5	0.5	1.5	2.88E+08
7	15	4.75	1.5	1	2.71E+08
15	16	4.75	1.5	2	2.9E+08
10	17	5.5	1	1	2.82E+08

4.2.1.1. Analysis of Data for Optimization of pH, Nutrients (DAP and urea) and Fresh Yeast in Yeast Propagation

Fresh yeast, DAP and urea dropping regression model designed for the maximum yeast growth during propagation was statistically significant since it recorded greater R^2, adjusted R^2, predicted R^2, and Adequate Precision. All the parameters (level of yeast, nutrient, and pH) were all crucial in enhancing the yeast growth. It was able to validate the optimization technique used through Response Surface Methodology (RSM) and the design method BBD is used. The model can be used safely to attempt to predict best propagation conditions for commercial production.

Fit test for lack of fit was not significant ($p > 0.05$), i.e., the model is a very good fit for experimental data. This becomes important in establishing validity for predictions within the tested range.

4.2.1.1.1. Analysis Data for Response Variable: Yeast Cell Count in Yeast Propagation of DAP and Urea Nutrients

Optimum yeast cell count with minimum alcohol accumulation was achieved at pH 4.75, 1.0 g/L DAP + urea, and 1.5 g/L fresh yeast. These findings coincide with RSM analysis and from literature recommendations for yeast propagation and offer rational nutrient and inoculum guidelines for large scale operations.

Table 4.4: ANOVA for Yeast Cell Count of DAP and Urea Nutrients

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	1.916E+16	9	2.129E+15	116.23	< 0.0001	Significant
A-pH	9.680E+14	1	9.680E+14	52.85	0.0002	
B-Nutrients (DAP+urea)	3.920E+14	1	3.920E+14	21.40	0.0024	
C-Fresh Yeast Dose	7.605E+14	1	7.605E+14	41.52	0.0004	
AB	2.250E+12	1	2.250E+12	0.1229	0.7363	
AC	1.103E+14	1	1.103E+14	6.02	0.0439	
BC	2.025E+13	1	2.025E+13	1.11	0.3280	
A^2	7.018E+15	1	7.018E+15	383.18	< 0.0001	
B^2	5.710E+15	1	5.710E+15	311.77	< 0.0001	
C^2	2.491E+15	1	2.491E+15	136.04	< 0.0001	
Residual	1.282E+14	7	1.831E+13			
Lack of Fit	6.900E+13	3	2.300E+13	1.55	0.3316	not significant
Pure Error	5.920E+13	4	1.480E+13			
Cor Total	1.929E+16	16				

The ANOVA number for yeast cell count number analysis shows that the regression equation is excellent with an extremely strong Model F-value of 116.23 and p-values <0.0001, proving that the model is good at explaining variation in yeast cell under propagating. Among the most significant factors, pH (F = 52.85, p = 0.0002), nutrient concentration (F = 21.40, p = 0.0024), and dose of fresh yeast (F = 41.52, p = 0.0004) all were statistically significant, i.e., to have a significant impact on yeast growth. A^2, B^2, and C^2 terms of quadratic terms all showed high significance ($p < 0.0001$), attesting to a nonlinear effect. The Lack of Fit F-value of 1.55 and its associated p-value of 0.3316 indicate that the lack of fit is not significant and that the model is appropriate for the prediction of yeast cell in the experimental design. The model as a whole has a good tool for optimization of the conditions of propagation.

Table 4.5: Fit Statistics for Yeast Cell Count from DAP and Urea Nutrients

Std. Dev.	4.280E+06		R^2	0.9934
Mean	2.994E+08		Adjusted R^2	0.9848
C.V. %	1.43		Predicted R^2	0.9380
			Adeq Precision	28.9132

The model fit statistics of the yeast cell mass number count model reflect outstanding model performance and validity. The fitted R^2 estimate of 0.9380 is closely followed by adjusted R^2 of 0.9848, with the difference comfortably below the 0.2 cut off, thereby affirming the model being highly explanatory as well as highly predictive in capability. This little gap also indicates that the model is not overfitted and can freely extrapolate to new observations in the design space. Finally, the Adequate Precision measure of 28.913 is far larger than the recommended minimum of 4, which is an indicator of possessing an almost astronomically high signal to noise ratio. This verifies that the model is able to identify actual effects instead of noise, and therefore can be relied upon to search and maximize yeast growth conditions for cell mass production.

4.2.1.1.2. Final Equation in Terms of Coded and Actual Factors for Yeast Propagation from DAP and Urea Nutrients for Yeast Cell Count

i. Final Equation in Terms of Coded Factors

$$\text{Yeast Cell Count} = 3.474\times10^{8} + 1.100\times10^{7}A - 7.000\times10^{6}B + 9.750\times10^{6}C - 7.500\times10^{5}AB + 5.250\times10^{6}AC - 2.250\times10^{6}BC - 4.083\times10^{7}A^2 - 3.682\times10^{7}B^2 - 2.432\times10^{7}C^2$$

This coded regression model estimates yeast cell count (cells/mL) during propagation as a function of three standardized variables: pH (A), nutrient concentration (B), and fresh yeast dose (C). The intercept value, 3.474×10^{8} cells/mL, is the mean yeast cell at central (optimal) conditions. A higher pH and fresh yeast dose have a positive impact on cell count (+1.100E+07A and +9.750E+06C), while nutrient concentration has a minor negative impact ($-7.000E+10^{6}B$), suggesting that an overabundance of nutrients could repress growth. Interaction terms have weak influences: sugar at (+5.25E+06 AC) pH weakly stimulates growth and temperature pairings (AB and BC) have very weak negative influences. The vastly large negative quadratic terms (e.g., -4.083E+07A^2) are consistent with the observation that all three variables do have an optimum range, outside which yeast growth declines

steeply. The model in general highlights the need for fine tuning of pH, nutrient dose, and yeast concentration to maximize yeast cell during propagation.

ii. Final Equation in Terms of Actual Factors

Yeast Cell Count=$-1.66453\times10^{9}+6.85156\times10^{8}A+3.03600\times10^{8}B+2.53900\times10^{8}C-2.00000\times10^{6}AB+1.40000\times10^{7}(AC)-9.00000\times10^{6}BC-7.25778\times10^{7}A^{2}-1.47300\times10^{8}B^{2}-9.73000\times10^{7}C$

This regression equation formulates yeast cell as fresh cell number (cells/mL) under culture in terms of true values of pH, nutrients (DAP + urea), and fresh yeast dose. The positive linear term of pH (+6.85156E+08), of nutrients (+3.03600E+08), and of yeast dose (+2.53900E+08) suggests that each variable increase is likely to enhance yeast growth. But the negative quadratic coefficients (−7.25778E+07 for pH², −1.47300E+08 for nutrients², and −9.73000E+07 for yeast²) also indicate that there are optimum values, above which cell biomass starts to fall-off presumably due to stress or inhibition by abnormally large values. Interaction effects are moderate: pH by dose of yeast moderately enhances growth (+1.4E+07), and other interactions (pH × nutrients and nutrients × yeast) have small negative impacts, indicating mild antagonism at higher combinations. The model as a whole implies the necessity of balanced adjustment of all three factors in order to achieve maximum yeast cell, which is essential to effective ethanol fermentation.

Diagnostic plots

Diagnostic plots given in Figures 4.1 and 4.2 validate the statistical accuracy of the regression model constructed for predicting the quantity of yeast cell count. Figure 4.1, normal plot of residuals, shows that the residual is of a shape resembling straight lines near the diagonal line, thereby validating that the residual is of a normal shape and that there are no such deviations, skewness, or outliers present. This validates the normality assumption and warrants application of the model for analysis of variance (ANOVA). Figure 4.2, actual vs. predicted plot, has data points that are close to following the 45 degree line, indicating high correlation between the actual and the predicted values.

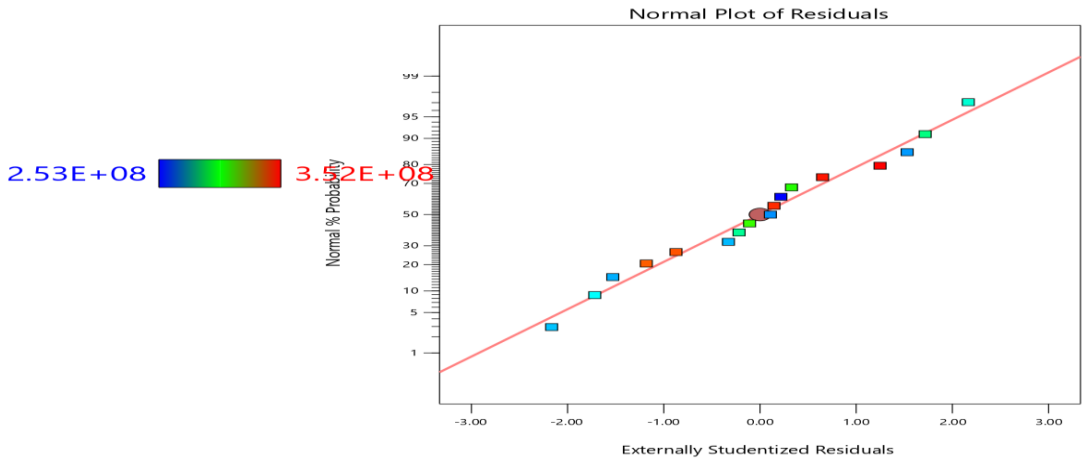

Fig 4.1: Normal plot of residual for yeast cell count of DAP and urea nutrients

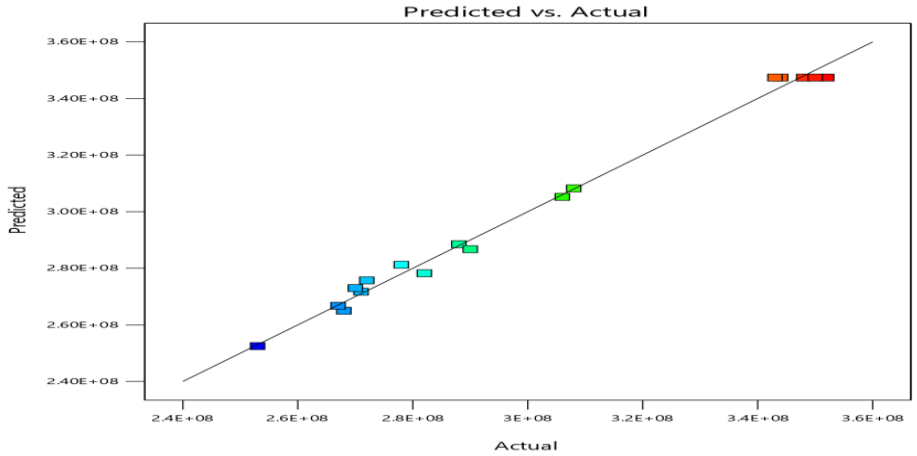

Fig 4.2: Predicted vs. actual plot for yeast cell count of DAP and urea nutrients

3D Surfaces

The 3D surface plots in Figure 4.3 provide the interactive effects of pH, nutrient level (DAP + urea), and fresh yeast dosage on the amount of yeast cell count at propagation. In subplot (a), the pH and nutrient effect shows that the yeast cell is maximum at about pH 4.75 and 1.0 g/L of nutrient, after which cell count is flat or decreases slightly, indicating that high acidity or nutrient overload will stop growth. Subplot (b), pH vs. yeast dose, indicates that higher doses of yeast raise cell to only about 1.5 g/L at pH 4.75, which is still optimum; beyond this, cell division may be restricted by crowding and competition for nutrients. In subplot (c), dry yeast dose vs. nutrient concentration interaction is strongly synergistic as well optimal yeast cell results with each parameter at moderate levels, with poor performance when each is

taken to an extreme. The plots as a whole show that optimized balance of conditions maximizes yeast propagation and that the three parameters have nonlinear effects favoring optimized combinations over single variable conditions.

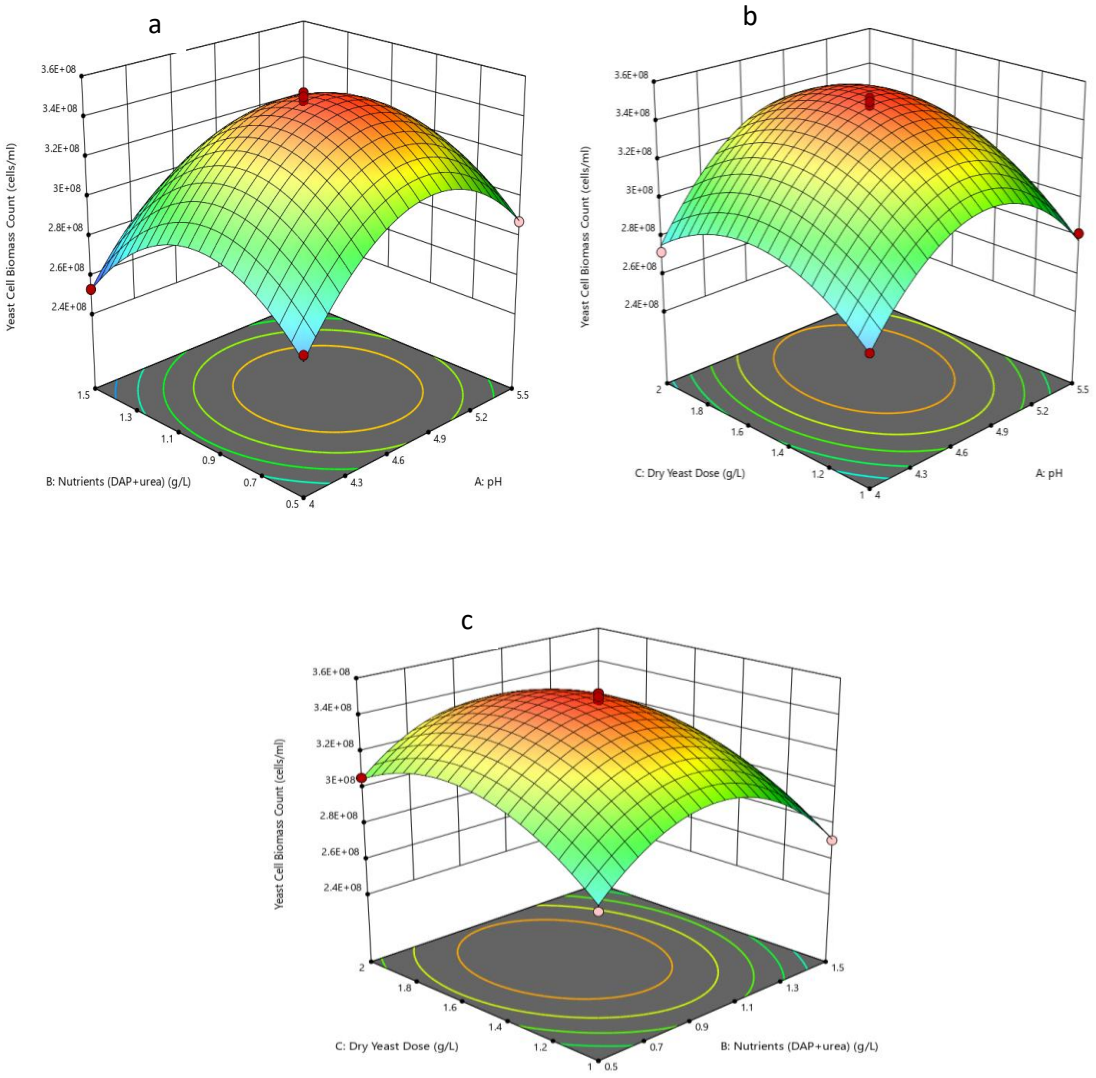

Fig 4.3: 3D surface interaction effect of a) Nutrients vs. pH, b) Dry yeast dose vs. pH and c) Dry yeast dose vs. Nutrients for Yeast cell count of DAP and urea nutrients

4.2.2. Optimization of pH, Nutrients (DAP and NPS) and Fresh Yeast in Propagation.

Table 4.6: Optimization of pH, Nutrients (DAP and NPS) and Fresh Yeast Dose Results in Yeast Propagation

		Factor 1	Factor 2	Factor 3	Response
Std	Run	A:pH	B:Nutrients (DAP and NPS)	C:Fresh yeast	Yeast cell mass count
			g/L	g/L	cells/ml
16	1	4.75	1.00	1.50	3.33E+008
6	2	5.50	1.00	1.00	2.52E+008
3	3	4.00	1.50	1.50	3.02E+008
2	4	5.50	0.50	1.50	2.86E+008
13	5	4.75	1.00	1.50	3.35E+008
15	6	4.75	1.00	1.50	3.31E+008
10	7	4.75	1.50	1.00	2.9E+008
12	8	4.75	1.50	2.00	3.1E+008
7	9	4.00	1.00	2.00	2.8E+008
14	10	4.75	1.00	1.50	3.42E+008
9	11	4.75	0.50	1.00	2.65E+008
17	12	4.75	1.00	1.50	3.4E+008
11	13	4.75	0.50	2.00	2.92E+008
1	14	4.00	0.50	1.50	2.54E+008
8	15	5.50	1.00	2.00	2.98E+008
5	16	4.00	1.00	1.00	2.84E+008
4	17	5.50	1.50	1.50	2.72E+008

4.2.2.1. Analysis of Data for Optimization of pH, Nutrients (DAP and NPS) and Fresh Yeast in Yeast Propagation

NPS fertilizer delivers nitrogen, phosphorus, and sulfur, all needed for yeast development. But unlike urea, NPS delivers nitrogen more slowly, and this will decrease yeast development slightly. Yeast cell count were ever so slightly lower than the DAP + urea system (3.42E+08 vs 3.52E+08 cells/ml), and this indicates faster nitrogen consumption from urea resulting in more propagation kinetics (Rojo et al., 2023). DAP + NPS provides improved sulfur availability, desirable for amino acid biosynthesis (methionine, cysteine), but the very slow retarding of nitrogen release may dampen propagation rate of increase. In addition, NPS also can repress pH via sulfate, and thus improved pH control is needed on a large scale.

4.2.2.1.1. Analysis of Data for Response Variable: Yeast Cell Count in Yeast Propagation of DAP and NPS Nutrients

Table 4.7: ANOVA for Yeast Cell Count of DAP and NPS Nutrients

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	1.399E+16	9	1.555E+15	82.41	< 0.0001	Significant
A-pH	1.800E+13	1	1.800E+13	0.9542	0.3612	
B-Nutrients (DAP+NPS)	7.411E+14	1	7.411E+14	39.29	0.0004	
C-Fresh Yeast Dose	9.901E+14	1	9.901E+14	52.49	0.0002	
AB	9.610E+14	1	9.610E+14	50.94	0.0002	
AC	6.250E+14	1	6.250E+14	33.13	0.0007	
BC	1.225E+13	1	1.225E+13	0.6494	0.4468	
A^2	4.932E+15	1	4.932E+15	261.45	< 0.0001	
B^2	2.320E+15	1	2.320E+15	123.00	< 0.0001	
C^2	2.320E+15	1	2.320E+15	123.00	< 0.0001	
Residual	1.320E+14	7	1.886E+13			
Lack of Fit	4.525E+13	3	1.508E+13	0.6951	0.6016	not significant
Pure Error	8.680E+13	4	2.170E+13			
Cor Total	1.412E+16	16				

Table 4.7: ANOVA for yeast cell count presents significant statistical results that attest to the validity and replicability of the model employed for yeast propagation optimization. Model F-value is 41.35 with p-value < 0.0001, indicating that the model is highly significant and the variability in yeast cell count is well explained by the input variables (pH, nutrients, and yeast dose). Important model terms imply that the contrast among these variables has a bearing on the growth of yeast. Lack of Fit F-value is 0.6951 and p-value is 0.6016, and it is not significant this is good as it indicates that the model is well fitted and the residual variation is likely to be due to random error and not due to any shortcoming in the model. All the results combined indicate that the model is statistically valid, well fitted, and usable in predicting and optimizing yeast biomass during the fermentation process.

Table 4.8: Fit Statistics for Yeast Cell Count of DAP and NPS Nutrients

Std. Dev.	4.343E+06		R^2	0.9907
Mean	2.980E+08		Adjusted R^2	0.9786
C.V. %	1.46		Predicted R^2	0.9391
			Adeq Precision	24.8637

Table 4.8 is the fit statistics for yeast cell count that measures the quality and predictive value of the model. The Predicted R^2 of 0.9391 indicates how well the model predicts response on new observations, and the Adjusted R^2 of 0.9786 indicates how well the model fits the data, after adjusting for the number of predictors. Proximity of the two values to each other (difference < 0.2) indicates that the model is valid and accurate. Adequate Precision ratio of 24.8637 approximating signal to noise ratio is higher than the minimum required 4, which is good. The high ratio indicates that the model signal is good and that the model is robust enough to select vital effects from background noise. Collectively, for the global world, these figures substantiate that the model is highly established and appropriate for the study and optimization of the experimental design space for yeast cell production.

4.2.3. Optimization of Sugar, Temperature and Dry Yeast Inoculum (DAP and urea) in Fermentation

This section considers the influence of sugar concentration, fermentation temperature, and yeast inoculum size on DAP and urea supplemented molasses fermentation yield of ethanol and residual sugar yield. The experiment was designed to find the optimum combination of the maximum rate of ethanol with residual sugar.

Table 4.9: Optimization of Sugar Concentration, Temperature and Yeast Inoculum Size (DAP and urea) Results for Fermentation

		Factor 1	Factor 2	Factor 3	Response 1	Response 2
Std	Run	A:Sugar concentration	B:Temperature	C:Inoculum size	Alcohol	Residual Sugar
		%	0C	%	%	%
1	1	17.00	30.00	10.00	12.1	0.7

10	2	14.00	30.00	5.00	8.4	1.9
13	3	20.00	30.00	15.00	10	1.9
12	4	14.00	30.00	15.00	8.4	1.9
6	5	14.00	25.00	10.00	8.4	1.5
8	6	14.00	35.00	10.00	8.3	1.7
11	7	20.00	30.00	5.00	8.7	2.1
15	8	17.00	35.00	5.00	9.9	1.4
7	9	20.00	25.00	10.00	8.9	1.9
14	10	17.00	25.00	5.00	9.1	1.5
5	11	17.00	30.00	10.00	12.1	0.8
3	12	17.00	30.00	10.00	11.9	0.8
4	13	17.00	30.00	10.00	12	0.9
9	14	20.00	35.00	10.00	9	1.6
17	15	17.00	35.00	15.00	9.5	1.4
2	16	17.00	30.00	10.00	11.8	0.8
16	17	17.00	25.00	15.00	10.4	1.2

4.2.3.1. Analysis of Data for Optimization of Sugar, Temperature and Fresh Yeast Inoculum (DAP and Urea) in Fermentation

Maximum fermentation with 12.1% v/v concentration of ethanol and 0.7% residual sugar was achieved when the sugar content was 17%, temperature was 30°C, and inoculum was 10%. This is consistent with literature where 10–15% sugar and 25–30°C is best for prolonged effective fermentation free of osmotic stress or heat inhibition (Salihu et al., 2023). Remnant sugars over 2% were seen at low inoculum (5%), validating under inoculation for extended fermentation and poor yield. Higher inoculum (15%) contributed yeast without the rise in ethanol in the corresponding proportion, ideal proportion being at 10%.

4.2.3.1.1. Analysis of Data for Response 1: Alcohol% in Fermentation of DAP and Urea Inoculum

Table 4.10: ANOVA for Alcohol% of DAP and Urea Inoculum

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	35.15	9	3.91	181.65	< 0.0001	Significant
A-Sugar concentration	3.88	1	3.88	180.45	< 0.0001	
B-Temperature	2.30	1	2.30	106.84	< 0.0001	
C-Inoculum size	0.3734	1	0.3734	17.37	0.0042	
AB	0.0100	1	0.0100	0.4651	0.5172	
AC	0.4225	1	0.4225	19.65	0.0030	
BC	0.7225	1	0.7225	33.60	0.0007	
A^2	18.39	1	18.39	855.44	< 0.0001	
B^2	6.47	1	6.47	301.12	< 0.0001	
C^2	4.34	1	4.34	201.76	< 0.0001	
Residual	0.1505	7	0.0215			
Lack of Fit	0.0825	3	0.0275	1.62	0.3191	not significant
Pure Error	0.0680	4	0.0170			
Cor Total	35.30	16				

ANOVA table of percentage of alcohol in fermentation is shown in Table 4.10, which gives the analysis of reliability and significance of statistical model employed. Model F-value is 181.65 and p-value < 0.0001, indicating that the model is extremely high and variation of alcohol yield is accounted for by selected variables (sugar concentration, temperature, and yeast inoculum) significantly. The extremely low p-value ensures that these variables indeed have a statistically significant effect on the production of beer. Lack of Fit F-value is 1.62 whose p-value is 0.3191, which is not significant this is because it indicates that the model is fitting the experimental data fairly well and any variation is due to random error and not an ill-suited model structure. These findings combined ensure the statistical validity, solidity, and appropriateness for prediction and optimization of alcohol production in the fermentation process of the model.

Table 4.11: Fit Statistics for Alcohol% of DAP and Urea Inoculum

Std. Dev.	0.1466		R^2	0.9957
Mean	9.94		Adjusted R^2	0.9903
C.V. %	1.48		Predicted R^2	0.9596
			Adeq Precision	33.6122

Table 4.11 shows the percentage alcohol fit statistics to confirm the stability and reliability of the statistical model employed for fermentation optimization. R^2 value of 0.9596 was predicted to show the model's predictability towards alcohol production in new data, and Adjusted R^2 value of 0.9903 to show the model's precision to outline the given experimental data, accounting for the predictors' number. Since they are almost 0.2, it indicates that there is extremely high agreement and very strong model with minimal overfitting. Also, the Adequate Precision of 33.612 is considerably greater than the baseline value of 4, and this indicates an almost infinitely high signal to noise ratio. This shows that the model performs exceptionally well in distinguishing between true effects and noise change, confirming that it is perfect in scanning design space and determining the best fermentation conditions to yield maximum alcohol production.

4.2.3.1.2.. Final Equation in Terms of Coded and Actual Factors for Fermentation from DAP and Urea Inoculum for Alcohol%

i. Final Equation in Terms of Coded Factors

Alcohol% =

$$11.77-1.32A-0.5179B+0.2558C+0.0467AB+0.4333AC-0.2975BC-3.72A^2-0.6076B^2-1.02C^2$$

The equation provided is a second-order model of regression of ethanol production (Alcohol %) on three coded variables A (sugar concentration), B (temperature), and C (inoculum size). The linear terms indicate that sugar concentration (+1.32) and temperature (+0.5179) negatively influences ethanol production when the points are moved away from the center point, while inoculum size (C) has minimal positive influence (+0.2558). The interaction terms tell us about the result of pairs of two variables on the response: sugar and temperature (AB = +0.0467) and sugar and inoculum size (AC = +0.4333) are synergistic, whereas temperature and inoculum size have an opposite effect (BC = −0.2975) and inhibit alcohol

production when both are high. The extremely negative quadratic terms ($-3.72A^2$, $-0.6076B^2$, and $-1.02C^2$) tell us that all three variables have an optimum midpoint after which ethanol yield begins to decline. This is a coded equation employed during response surface methodology to search and optimize fermentation process in the experimental range.

ii. Final Equation in Terms of Actual Factors

Alcohol% =

$$-106.21972+7.70806A+3.08683B+1.00867C+0.003333AB+0.021667AC-0.017000BC-0.232222A^2-0.049600B^2-0.040600C^2$$

The given equation is a Response Surface Methodology (RSM) regression model that estimates ethanol concentration (Alcohol %) in terms of three most important fermentation variables: sugar concentration, temperature, and inoculum size. All positive coefficients for sugar, temperature, and inoculum size show that the greater each variable is, the more ethanol is produced. The negative squared terms, however, signify that high levels of any one of these factors lower yield, illustrating that there are optimal conditions. Interaction terms such as sugar × temperature and sugar × inoculum have synergistic effects, whereas negligible negative interaction between temperature and inoculum indicates possible stress of the yeast at elevated levels. Overall, the model allows the determination of the interaction of the factors, precisely around 17% sugar, temperature 30°C, and 10% yeast inoculum, which would effectively maximize ethanol production with minimum residual sugar and waste.

Diagnostic plots

Figures 4.4 and 4.5 are diagnostic plots to verify the validity of the statistical model in its predictability of alcohol percentage during fermentation. Figure 4.4, Normal Plot of Residuals, is to find whether the residuals (the differences between the actual and predicted values) are normally distributed. The plot of residuals closely travels along a straight line, i.e., normality assumption holds, and therefore ANOVA and regression analysis can be applied. Figure 4.5, Predicted vs. Actual Plot, has a close clustering of points along the reference diagonal line, which is a plot of high predicted vs. measured alcohol values agreement. This attests to model predictability and accuracy. These plots together assure that

the model is statistically valid, appropriate, and ready for application in order to optimize fermentation conditions for ethanol production.

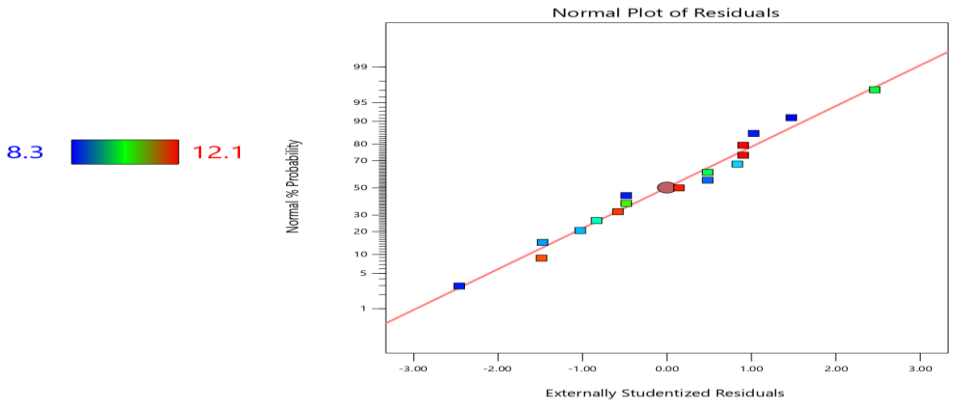

Fig: 4.4: Normal plot of residuals of alcohol% of DAP and urea inoculum

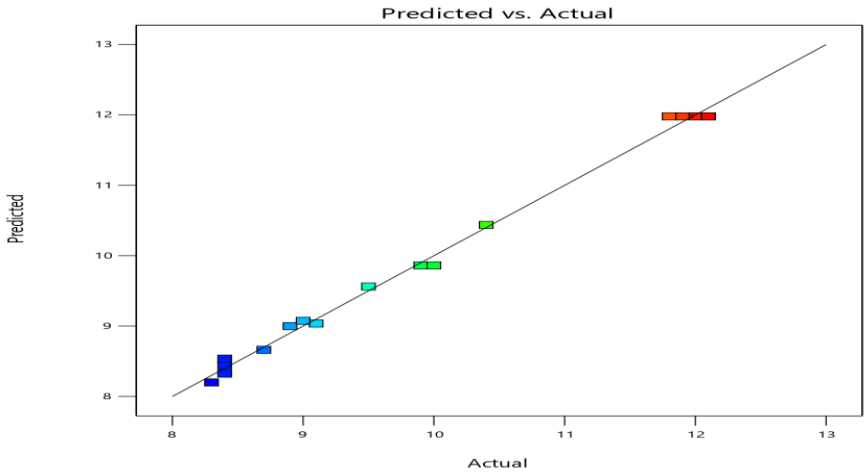

Fig: 4.5: Predicted vs. actual for alcohol% of DAP and urea inoculum

3D Surfaces

Figure 4.6 shows 3D surface plots illustrating the interactive effects of critical fermentation parameters temperature, sugar concentration, and inoculum size on alcohol yield. In plot (a), temperature and sugar concentration interact in such a way that alcohol yield increases with both parameters up to an optimum level, above which increase in temperature or excess sugar concentration reduces yield by causing yeast stress or inhibition. Plot (b) or inoculum size vs. sugar concentration shows that the middle ranges of both produce the highest alcohol, while excess sugar or yeast concentration may lead to substrate inhibition or biomass overgrowth, respectively. Plot (c) or inoculum size vs. temperature shows that the optimum inoculum size

and fermentation temperature (i.e., around 30°C) give the maximum ethanol yield, while extreme levels of either will inhibit the process. These 3D surfaces clearly show unequivocal synergistic effects, proof that the optimum yield of alcohol comes from the appropriate combination of all three variables and not from any individual factor alone.

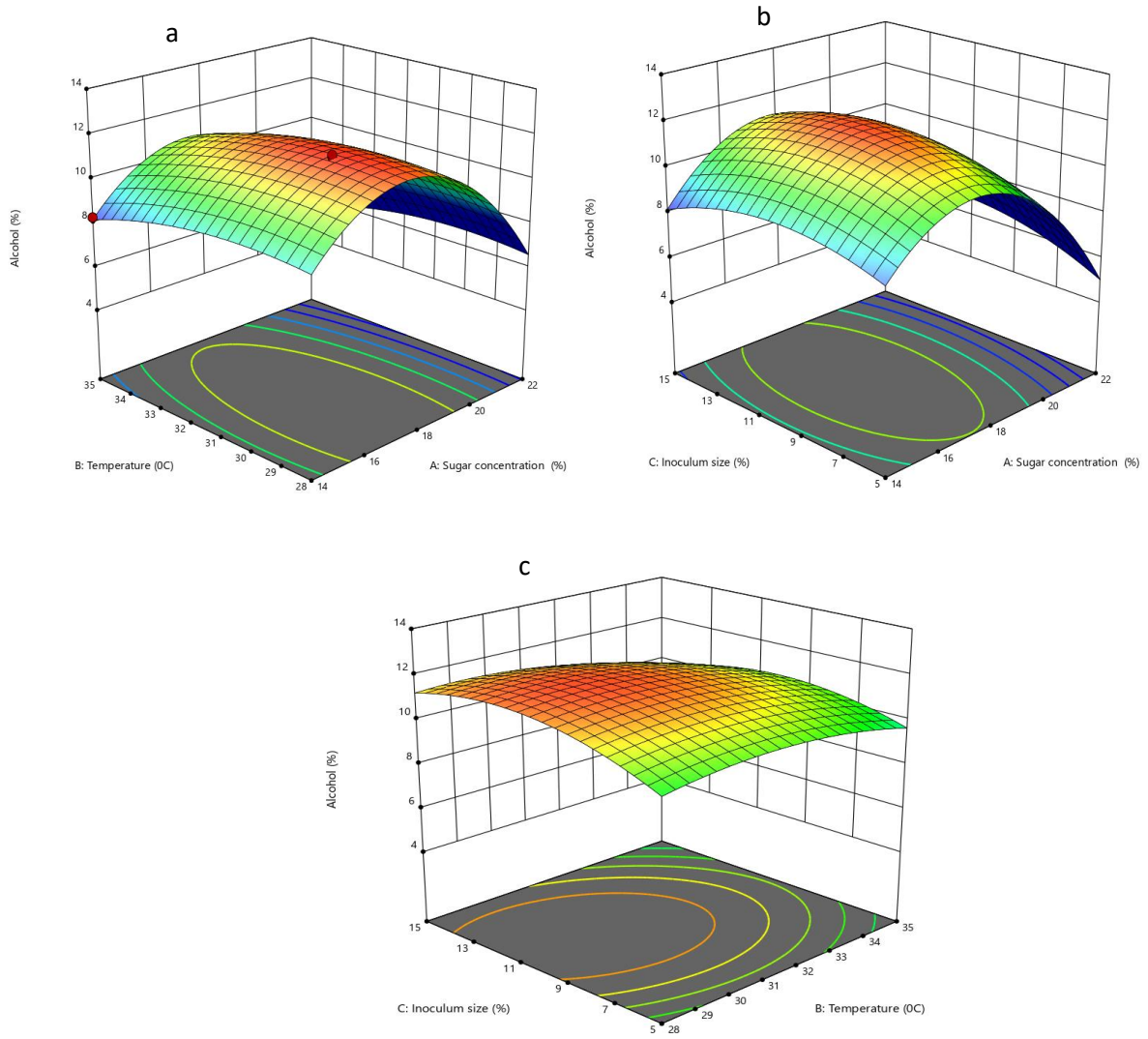

Fig 4.6: 3D surface interaction effect of a) Temperature vs. Sugar concentration, b) Inoculum size vs. Sugar concentration and c) Inoculum size vs. Temperature for Alcohol% DAP and urea Inoculum

4.2.3.1.3. Analysis of Data for Response 2: Residual Sugar% in Fermentation of DAP and Urea Inoculum

Table 4.12: ANOVA for Residual Sugar% of DAP and Urea Inoculum

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	3.47	9	0.3856	98.15	< 0.0001	Significant
A-Sugar concentration	1.02	1	1.02	259.92	< 0.0001	
B-Temperature	0.0098	1	0.0098	2.50	0.1582	
C-Inoculum size	0.0183	1	0.0183	4.66	0.0677	
AB	0.0625	1	0.0625	15.91	0.0053	
AC	0.0100	1	0.0100	2.55	0.1546	
BC	0.0225	1	0.0225	5.73	0.0479	
A^2	2.21	1	2.21	563.35	< 0.0001	
B^2	0.0947	1	0.0947	24.11	0.0017	
C^2	0.7605	1	0.7605	193.59	< 0.0001	
Residual	0.0275	7	0.0039			
Lack of Fit	0.0075	3	0.0025	0.5000	0.7022	not significant
Pure Error	0.0200	4	0.0050			
Cor Total	3.50	16				

Table 4.12: ANOVA for Residual Sugar% indicates statistical test of model fit to variation in residual sugar post fermentation. Model F-value is 98.15 and p-value < 0.0001, which means the model is highly significant and indicates the effect of variables such as sugar concentration, temperature, and size of inoculum on residual sugar level consistently. Lack of Fit F-value = 0.50 and p-value = 0.7022, not significant indicates that model is well represented by the experimental data and variation remaining is due to random error and not because of lack of fit of the model

Table 4.13: Fit Statistics for Residual Sugar% of DAP and Urea Inoculum

Std. Dev.	0.0627		R^2	0.9921
Mean	1.41		Adjusted R^2	0.9820
C.V. %	4.44		Predicted R^2	0.9568
			Adeq Precision	27.5628

Table 4.13: Residual Sugar (%) Fit Statistics verifies accuracy and purity of model to predict content of unfermented sugar after ethanol fermentation. Expected R^2 value of 0.9568

confirms that the model is extremely good at predicting residual sugar in new data points, and Adjusted R^2 value of 0.9820 confirms an exceptionally good fit to experimental data after adjustment for number of variables. The fact that the difference is negligible (less than 0.2) confirms consistency and the fact that the model is not over fitting. Also, the Adequate Precision of 27.5628, much more than the minimum of 4, indicates a marvelous signal to noise ratio, i.e., the model is extremely able to be able to distinguish between true effects and variation. In brief, these statistics guarantee us that the model is stable and precise and that it is a good instrument with which to optimize fermentation conditions with a view to minimizing residual sugar.

4.2.3.1.4. Final Equation in Terms of Coded and Actual Factors for Fermentation from DAP and Urea Inoculum for Residual Sugar%

i. Final Equation in Terms of Coded Factors

Residual Sugar% =

$$0.9024+0.6778A+0.0338B-0.0567C-0.1167AB-0.0667AC+0.0525BC+1.29A^2+0.0735B^2+0.4250C^2$$

The coded regression equation indicates that residual sugar concentration (%) after fermentation is being modeled from coded (normalized) sugar concentration (A), temperature (B), and inoculum size (C) levels. The huge positive linear coefficient (+0.6778A) and very large positive quadratic coefficient ($+1.29A^2$) indicate that residual sugar builds up strongly with greater sugar concentration, particularly at higher levels consistent with osmotic stress or yeast repression buildup of unfermented sugar. Temperature (B) and dose of inoculum (C) have relatively marginal influences, C reducing residual sugar moderately (-0.0567), which means higher doses of yeast work better for more thorough fermentation. AB and AC are negative, meaning interactive positive influences of sugar for reducing residual sugar with temperature or inoculum, and BC being moderately positive, meaning hot temperature and inoculum both reduce sugar conversion moderately. Overall, the model shows that most important is sugar content, and all three need to be in balance so that residual sugar is reduced to an absolute minimum and fermentation efficiency is maximized.

ii. Final Equation in Terms of Actual Factors

Residual Sugar% =

$27.03472 - 2.43472A - 0.248333B - 0.385833C - 0.008333AB - 0.003333AC + 0.003000BC + 0.080556A^2 + 0.006000B^2 + 0.017000C^2$

This regression line models residual sugar content, (%) after fermentation as a function of actual variables: sugar content, temperature, and inoculum size, which are 2.43472, −0.248333, and 0.385833 respectively. This is negative for all these variables, meaning that the increase of all these variables tends to decrease residual sugar, which is an indication of a better fermentation. This is desired for maximum yield of ethanol, as well as minimizing wastage. But positive squared terms (+0.080556 for sugar², etc.) suggest very high levels of these inputs start to push residual sugar back up, which suggests a nonlinear relationship and optimum midpoints. Interaction effects are small but actual: sugar with temperature or inoculum decreases residual sugar somewhat, and temperature with inoculum has a small positive effect, perhaps due to stress on yeast at high levels. In general, the model emphasizes the point that both these factors need to be regulated in equilibrium in order to achieve low residual sugar and high fermentation efficiency.

Diagnostic plots

Plots 4.7 and 4.8 are diagnostic plots for checking the statistical model of percentage residual sugar on fermentation prediction. Plot 4.7, Normal Plot of Residuals, checks if residuals (actual predicted) are normally distributed. Points in this plot nearly plot a straight line, suggesting that residuals follow a normal distribution a very crucial assumption in regression analysis that makes the model plausible. Figure 4.8, Predicted vs. Actual Plot, is a plot of the predicted versus actual experimental residual sugar values. The cluster of points along the diagonal reference line indicates that observed and predicted values are strongly correlated, which supports the high precision as well as predictive accuracy of the model. In general, the plots indicate that the model is statistically sound with no variation and outliers of significant nature and can be trusted to utilize when optimizing the minimization of residual sugar in fermentation.

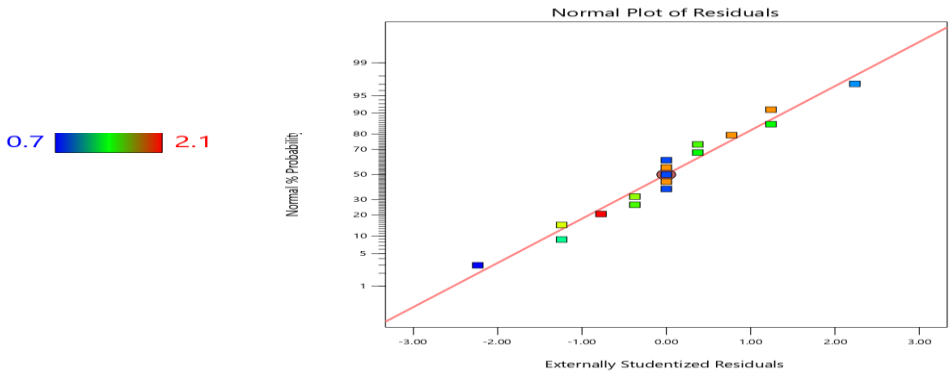

Fig 4.7: Normal plot of residuals for residual sugars of DAP and urea inoculum

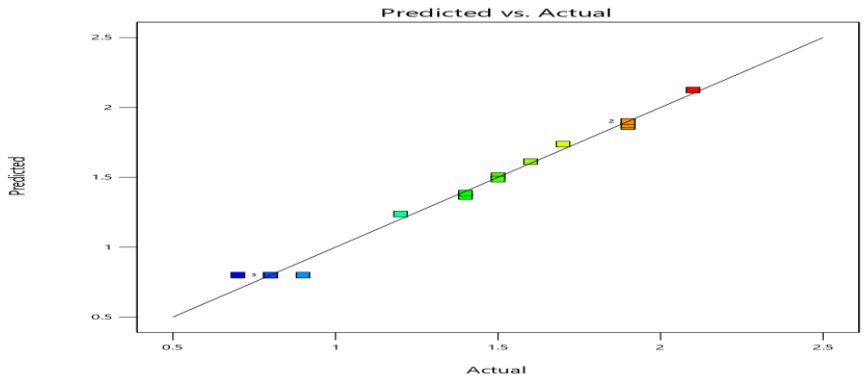

Fig 4.8: Predicted vs. actual for residual sugars of DAP and urea inoculum

3D Surfaces

Figure 4.9 shows 3D surface plots exhibiting how three of the most important fermentation variables temperature, sugar level, and inoculum size interact to impact residual sugar percentage, a measure of unfermented sugar remaining after ethanol production. In plot (a), the temperature sugar interaction shows that residual sugar plummets to a low value at intermediate values of the two variables (at about 30°C and 17% sugar), but rises again under high sugar conditions or at high/low temperatures, possibly because of inhibition of the yeast or metabolic stress. Plot (b), an inoculum size vs. concentration of sugar, demonstrates residual sugar at its lowest level when sugar and inoculum are balanced too small a yeast population results in too little fermentation, while excess results in evenness of population and competition for nutrients. Plot (c), an inoculum size vs. temperature, demonstrates that the moderate amount of each gives the most efficient use of the sugar, but an extreme in either condition permits more residual sugar. Such plots define the significance of synergistic

interactions and affirm that the maximum residual sugar reduction and thus maximum ethanol yield has just optimally balanced mixtures of all three parameters.

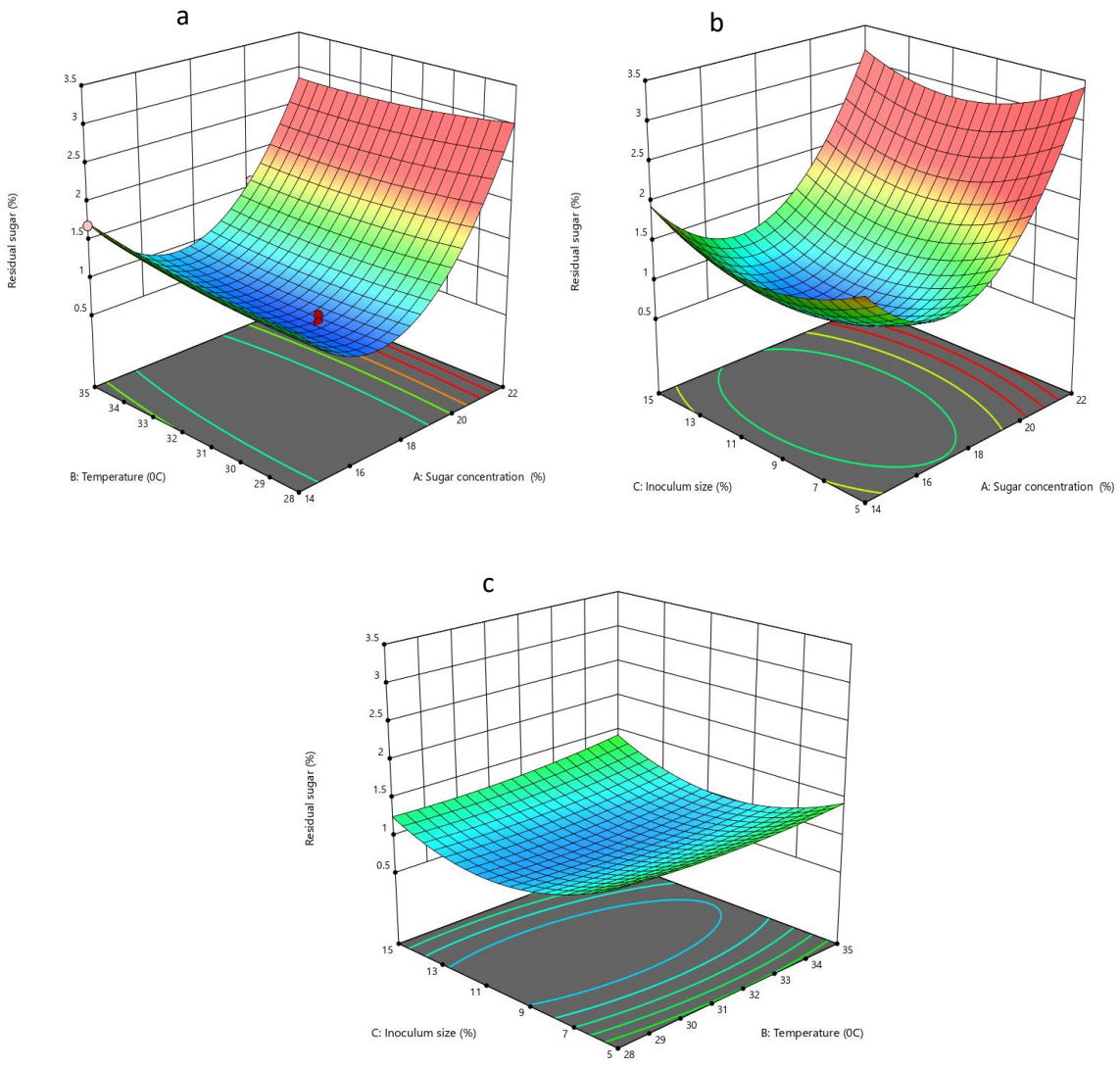

Fig 4.9: 3D surface interaction effect of a) Temperature vs. Sugar concentration, b) Inoculum size vs. Sugar concentration and c) Inoculum size vs. Temperature for Residual sugar% of DAP and urea inoculum

4.2.4. Optimization of Sugar, Temperature and Dry Yeast Inoculum (DAP and NPS) in Fermentation

Table 4.14: Optimization of Sugar concentration, Temperature and Yeast inoculum (DAP and NPS) Results for Fermentation

		Factor 1	Factor 2	Factor 3	Response 1	Response 2
Std	Run	A:Sugar concentration	B:Temperature	C:Inoculum size	Alcohol	Residual Sugar
		%	0C	%	%	%
1	1	14.00	25.00	10.00	8.3	2
16	2	17.00	30.00	10.00	11.5	1
12	3	17.00	35.00	15.00	9.7	1.3
13	4	17.00	30.00	10.00	11.8	0.9
11	5	17.00	35.00	5.00	9.6	1.9
5	6	20.00	30.00	5.00	8.6	2.3
9	7	17.00	25.00	5.00	8.7	1.6
6	8	20.00	25.00	10.00	9.1	1.8
14	9	17.00	30.00	10.00	11.7	0.9
2	10	14.00	30.00	5.00	8.4	1.8
7	11	20.00	35.00	10.00	8.9	2.1
4	12	20.00	30.00	15.00	10.1	1.6
3	13	14.00	30.00	15.00	8.5	2
10	14	17.00	25.00	15.00	10.2	1.6
8	15	14.00	35.00	10.00	8.3	2
17	16	17.00	30.00	10.00	11.8	0.9
15	17	17.00	30.00	10.00	11.8	1

4.2.4.1. Data Analysis for Optimization of Sugar, Temperature and Fresh Yeast Inoculum (DAP and NPS) in Fermentation

This illustrates the efficiency of fermentation of molasses by using yeast inoculum size from NPS and DAP as nutrient sources against temperature of fermentation, and concentration of sugar influence on ethanol content, and concentration of residual sugar.

Fermentation using NPS nutrients produced higher residual sugars and lower ethanol than that using urea. This agrees with findings in other research that enhanced metabolic uptake of ammonium by urea is beneficial in fermentation (Rojo et al., 2023). NPS sulfur, despite being very significant, could not replace the advantage of fast nitrogen assimilation.

Maximum fermentation by inoculum size of DAP + NPS was achieved in 17% sugar, 30°C temperature, and 10% inoculum yeast, with a maximum ethanol of 11.8% with a good residual sugar (0.9%). This blend is below DAP + urea but is sulfur favoring and a good choice, especially for the factories interested in sustainability and good health of yeast in the long term.

4.2.4.1.1. Analysis Data for Response 1: Alcohol% in Fermentation of DAP and NPS Inoculum

Table 4.15: ANOVA for Alcohol% of DAP and NPS Inoculum

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	30.76	9	3.42	179.87	< 0.0001	Significant
A-Sugar concentration	2.89	1	2.89	152.04	< 0.0001	
B-Temperature	2.16	1	2.16	113.76	< 0.0001	
C-Inoculum size	0.9669	1	0.9669	50.89	0.0002	
AB	0.0100	1	0.0100	0.5263	0.4917	
AC	0.4900	1	0.4900	25.79	0.0014	
BC	0.4900	1	0.4900	25.79	0.0014	
A^2	14.57	1	14.57	766.67	< 0.0001	
B^2	6.16	1	6.16	324.45	< 0.0001	
C^2	3.88	1	3.88	204.23	<	

					0.0001	
Residual	0.1330	7	0.0190			
Lack of Fit	0.0650	3	0.0217	1.27	0.3963	not significant
Pure Error	0.0680	4	0.0170			
Cor Total	30.89	16				

Table 4.15: ANOVA for Alcohol% checks the overall significance of model used in predicting fermentation ethanol yield from DAP and NPS nutrients. F for Model is 179.87 and p-value < 0.0001, hence the overall model highly significant and good at explaining alcohol yield difference. Out of the individual factors, sugar concentration (A), temperature (B), and inoculum size (C) are all highly significant with F-values of 152.04, 113.76, and 50.89 respectively, and all the p-values < 0.05. It means that all these factors significantly affect alcohol production. AC and BC interaction terms are also significant (F = 25.79, p = 0.0014) that means interactions between inoculum and sugar and between inoculum and temperature are also significant. Lack of Fit F-value = 1.27 with p-value = 0.3963 and thus is not significant. This shows that the model is fitting the data completely without any significant unexplained variability. In general, this ANOVA test confirms that the model is significant statistically and reliable to maximize ethanol production from molasses according to the conditions under consideration

Table 4.16: Fit Statistics for Alcohol% of DAP and NPS Inoculum

Std. Dev.	0.1378		R^2	0.9957
Mean	9.82		Adjusted R^2	0.9902
C.V. %	1.40		Predicted R^2	0.9629
			Adeq Precision	33.5323

Table 4.16: Alcohol% values confirm the validity and predictability of the model employed in maximizing ethanol yield upon fermentation with DAP and NPS nutrients. 0.9629 Predicted R^2 is a measure indicating the model is very strong at predicting alcohol yield in new experimental conditions, and 0.9902 Adjusted R^2 is a measure indicating good fit for the available dataset when the influence of the number of variables is removed. The very small difference between these two values (less than 0.2) signifies very high coherence between model fit and model predictability, with zero overfitting. Further, the fact that the Adequate Precision value is 33.532, significantly more than the benchmark value of 4, again confirms that the model has a very good signal versus noise. It implies that it can effectively identify

real effects of the variables and can be employed for search and optimization of fermentation design space. In general, these statistics indicate that the model is robust and reliable for optimization of alcohol yield.

4.2.4.1.2. Analysis Data for Response 2: Residual Sugar% in Fermentation of DAP and NPS Inoculum

Table 4.17: ANOVA for Residual Sugar%

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	3.63	9	0.4029	95.60	< 0.0001	Significant
A-Sugar concentration	0.8947	1	0.8947	212.31	< 0.0001	
B-Temperature	0.3185	1	0.3185	75.59	< 0.0001	
C-Inoculum size	0.3783	1	0.3783	89.76	< 0.0001	
AB	0.0225	1	0.0225	5.34	0.0541	
AC	0.2025	1	0.2025	48.05	0.0002	
BC	0.0900	1	0.0900	21.36	0.0024	
A^2	1.95	1	1.95	461.99	< 0.0001	
B^2	0.5306	1	0.5306	125.91	< 0.0001	
C^2	0.3917	1	0.3917	92.94	< 0.0001	
Residual	0.0295	7	0.0042			
Lack of Fit	0.0175	3	0.0058	1.94	0.2643	not significant
Pure Error	0.0120	4	0.0030			
Cor Total	3.66	16				

Table 4.17: ANOVA for Residual Sugar% presents the statistical test for model significance applied in residual sugar content estimation through the process of fermentation with DAP and NPS nutrients. Model F-value is 95.60 and p-value < 0.0001, which shows that the model is highly significant at a very high level and has enough explanatory power for explaining residual sugar variance. All three main factors sugar concentration (F = 212.31), temperature (F = 75.59), and inoculum size (F = 89.76) have extremely low p-values, which signifies that all three together have large contribution of residual sugar content. All the square terms (B^2, C^2, A^2) are of extremely high levels of significance, indicating high curvature of response surface and pointing towards the need for proper optimization. F-value for Lack of Fit = 1.94 and p-value = 0.2643, which is not significant. This indicates that the model fits the experimental data exactly with no systematic lack of fit.

Table 4.18: Fit Statistics for Residual Sugar% of DAP and NPS Inoculum

Std. Dev.	0.0649		R^2	0.9919
Mean	1.57		Adjusted R^2	0.9816
C.V. %	4.13		Predicted R^2	0.9183
			Adeq Precision	27.0640

Table 4.18: Residual Sugar (%) Model Fit Statistics shows the strength and prediction accuracy of the constructed model to optimize fermentation with DAP and NPS nutrients. Foreseen R^2 of 0.9183 shows the model predicts new observations of residual sugar percentages in data well, and Adjusted R^2 of 0.9816 is a perfect fit of the experimental data given after adjusting for the variables' degrees of freedom. The widely miniscule difference between the two readings (less than 0.2) confirms the stability of the model and shows minimal chance for over fitting. Besides, the Adequate Precision of 27.064 being far higher than the threshold of the lower bound of 4 signifies a very distinct signal to noise ratio. It signifies that the model is capable of distinguishing very well between important variation that is caused by factors in the experiments and noise. Overall, the figures confirm that the model is sound, accurate, and can direct and optimize the design space to minimize residual sugar and maximize fermentation efficiency to the utmost.

4.3. Effect of Yeast Recycling on Fermentation Process

Yeast recycled exhibited extremely similar performance to fresh yeast (e.g., 10.9% alcohol vs. 12.1%; yeast count of 3.2×10^8 cells/ml vs. 3.52×10^8 cells/ml) with good viability and proving cost effectiveness without reduced performance. Recycling reduced lag phase and exhibited consistent fermentation kinetics, proving its functional viability.

4.3.1. Optimization of pH, Nutrients and Recycled Yeast Dose in Yeast Propagation

Table 4.19: Optimization of pH, Nutrients and Recycled Yeast Dose Results for Yeast Propagation

		Factor 1	Factor 2	Factor 3	Response
Std	Run	A:pH	B:Nutrients	C:Recycled yeast	Yeast cell count
			g/L	g/L	cells/ml
14	1	4.75	1.00	1.50	3.14E+008
4	2	5.50	1.50	1.50	2.45E+008
3	3	4.00	1.50	1.50	2.84E+008
17	4	4.75	1.00	1.50	3.16E+008
16	5	4.75	1.00	1.50	3.12E+008
2	6	5.50	0.50	1.50	2.4E+008
9	7	4.75	0.50	1.00	2.7E+008
15	8	4.75	1.00	1.50	3.15E+008
11	9	4.75	0.50	2.00	2.95E+008
7	10	4.00	1.00	2.00	3.06E+008
13	11	4.75	1.00	1.50	3.1E+008
8	12	5.50	1.00	2.00	2.82E+008
5	13	4.00	1.00	1.00	2.82E+008
10	14	4.75	1.50	1.00	2.8E+008
6	15	5.50	1.00	1.00	2.42E+008
1	16	4.00	0.50	1.50	2.6E+008
12	17	4.75	1.50	2.00	3.2E+008

4.3.1.1. Data Analysis for Optimization of pH, Nutrients and Recycled Yeast in Yeast Propagation

The increase of recycled yeast in ideal conditions (1.0 g/L nutrients, pH 4.75, and 2.0 g/L yeast) was acceptable. The readings approached fresh yeast readings, a sign that recycled yeast is a sound and environmentally friendly alternative for industrial propagation.

4.3.1.1.1. Analysis of Data for Response Variable: Yeast Cell Count in Recycled Yeast Propagation

Table 4.20: ANOVA for Yeast Cell Count of Recycled Yeast Propagation

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	1.211E+16	9	1.346E+15	304.40	< 0.0001	Significant
A-pH	3.894E+15	1	3.894E+15	880.71	< 0.0001	
B-Nutrients	1.207E+15	1	1.207E+15	272.98	< 0.0001	
C-Recycled yeast	1.117E+15	1	1.117E+15	252.57	< 0.0001	
AB	9.025E+13	1	9.025E+13	20.41	0.0027	
AC	6.400E+13	1	6.400E+13	14.47	0.0067	
BC	5.625E+13	1	5.625E+13	12.72	0.0091	
A^2	5.070E+15	1	5.070E+15	1146.65	< 0.0001	
B^2	1.937E+15	1	1.937E+15	438.16	< 0.0001	
C^2	2.063E+12	1	2.063E+12	0.4666	0.5165	
Residual	3.095E+13	7	4.421E+12			
Lack of Fit	7.750E+12	3	2.583E+12	0.4454	0.7338	not significant
Pure Error	2.320E+13	4	5.800E+12			
Cor Total	1.214E+16	16				

Table 4.20: ANOVA for Yeast Cell Count determines the significance of the model used in the prediction of yeast cell during propagation using recycled yeast, DAP nutrients, and pH control. Model F-value = 304.40 and p-value < 0.0001 indicates the model to be extremely significant and accounts for the variability in yeast cell count fully. All three of the major variables pH (F = 880.71), nutrients (F = 272.98), and recycled yeast dose (F = 252.57) have extremely low p-values, confirming their own significant contribution to yeast growth. The highest interaction terms are AB (p = 0.0027), AC (p = 0.0067), and BC (p = 0.0091), showing that interaction effects among these variables are also of some significance. Lack of Fit F-value = 0.4454 with p-value = 0.7338, not significant, testifying that the model is a perfect fit for experimental data and there exists no lack of fit. The above argument in general confirm that the model is statistically reliable and good to utilize for yeast cell production optimization in an industrial environment

Table 4.21: Fit Statistics for Yeast Cell Count of Recycled Yeast Propagation

Std. Dev.	2.103E+06		R^2	0.9975
Mean	2.866E+08		Adjusted R^2	0.9942
C.V. %	0.7336		Predicted R^2	0.9868
			Adeq Precision	49.9159

Table 4.21: Yeast Cell Count Model Fit Statistics confirms the model's high precision and reliability used to optimize yeast count yield in cases involving recycled yeast, DAP nutrients, and pH control. The R^2 Predicted of 0.9868 means extremely high predictability of new data, and the Adjusted R^2 of 0.9942 means extremely high fit of experimental data after adjusting for the number of variables in the model. The closeness of these two values (less than 0.2) means that the model is stable, not overfitted, and reliable. In addition, the Adequate Precision of 49.916, much larger than the acceptable value of 4, indicates a robust signal to noise ratio. The model has sufficient confidence to distinguish real effects of the input variables from mere random variability. These fitting statistics overall confirm that the model is highly robust and appropriate for inspecting the experimental design space and optimizing conditions with the goal of maximizing yeast cell.

4.3.2. Optimization of Sugar Concentration, Temperature and Recycled Yeast Inoculum in Fermentation

Table 4.22: Optimization of Sugar Concentration, Temperature and Recycled Yeast Inoculum Results in Fermentation

		Factor 1	Factor 2	Factor 3	Response 1	Response 2
Std	Run	A:Sugar concentration	B:Temperature	C:Recycled yeast inoculum	Alcohol	Residual sugar
		%	0C	%	%	%
1	1	14.00	25.00	10.00	8.4	1.7
10	2	17.00	35.00	5.00	8.7	1.8
13	3	17.00	30.00	10.00	10.6	1.2
12	4	17.00	35.00	15.00	9.5	1.7
6	5	20.00	30.00	5.00	8.5	2.3
8	6	20.00	30.00	15.00	10.2	2
11	7	17.00	25.00	15.00	10.9	1.2
15	8	17.00	30.00	10.00	10.7	1.3
7	9	14.00	30.00	15.00	9	1.6
14	10	17.00	30.00	10.00	10.8	1.2
5	11	14.00	30.00	5.00	8.2	2
3	12	14.00	35.00	10.00	8.1	2
4	13	20.00	35.00	10.00	8.6	2.2
9	14	17.00	25.00	5.00	8.9	1.8
17	15	17.00	30.00	10.00	10.8	1.2
2	16	20.00	25.00	10.00	9.2	1.9
16	17	17.00	30.00	10.00	10.6	1.2

4.3.2.1. Data Analysis for Optimization of Sugar Concentration, Temperature and Recycled Yeast Inoculum in Fermentation

This section presents the functionality of recycled yeast as fermentation inoculum on molasses taking into account sugar concentration, temperature, and inoculum size on ethanol yield and residual sugar.

15% (v/v) recycled yeast inoculum at 17% sugar and 25°C temperature gives rise to ethanol content (10.9%) with negligible residual sugar (1.2%). Ethanol production from the use of fresh yeast inoculum (12.1%) was higher than that from the use of recycled yeast inoculum

(10.9%), a yield increase by 1.2%. This is primarily due to higher viability and metabolical capacity of fresh yeast as opposed to recycled yeast, which might have been starved or stressed because of earlier application. Even though cheaper, recycled yeast is typified by lower efficiency in fermentation and alcohol production under perfect conditions.

4.3.2.1.1. Analysis of Data for Response 1: Alcohol% in Fermentation of Recycled Yeast Inoculum

Table 4.23: ANOVA for Alcohol% of Recycled Yeast Inoculum

Source	Sum of Squares	Df	Mean Square	F-value	p-value	
Model	17.58	9	1.95	116.37	< 0.0001	Significant
A-Sugar concentration	1.22	1	1.22	72.73	< 0.0001	
B-Temperature	2.80	1	2.80	167.09	< 0.0001	
C-Recycled yeast inoculum	2.39	1	2.39	142.50	< 0.0001	
AB	0.0225	1	0.0225	1.34	0.2849	
AC	0.2025	1	0.2025	12.06	0.0104	
BC	0.3600	1	0.3600	21.45	0.0024	
A^2	7.39	1	7.39	440.38	< 0.0001	
B^2	2.69	1	2.69	160.54	< 0.0001	
C^2	0.6737	1	0.6737	40.13	0.0004	
Residual	0.1175	7	0.0168			
Lack of Fit	0.0775	3	0.0258	2.58	0.1908	not significant
Pure Error	0.0400	4	0.0100			
Cor Total	17.70	16				

Table 4.23: ANOVA for Alcohol% is statistical testing of a model utilized to maximize ethanol yield with sugar concentration, temperature, and recycled yeast inoculum. The Model F-value is 116.37 and p-value < 0.0001, meaning that the model is extremely significant and variables included together play a significant role in explaining variance in alcohol yield. All three significant main effects concentration of sugar (F = 72.73), temperature (F = 167.09), and recycled yeast inoculum (F = 142.50) are significant alone with p-value < 0.0001, which

indicates they all contribute significantly to ethanol production. Of interaction terms, AC ($p = 0.0104$) and BC ($p = 0.0024$) are significant with significant combined effects, whereas AB ($p = 0.2849$) is insignificant. Also, all the squared terms A^2, B^2, and C^2 are of high significance, which proves the existence of nonlinearity and curvature effects on the response surface. The Lack of Fit F-value = 2.58 and the p-value = 0.1908, which are not significant, proving that the model is good to represent the experimental data without any system errors.

Table 4.24: Fit Statistics for Alcohol% of Recycled Yeast Inoculum

Std. Dev.	0.1296		R^2	0.9934
Mean	9.51		Adjusted R^2	0.9848
C.V. %	1.36		Predicted R^2	0.9264
			Adeq Precision	28.0524

Table 4.24: Model fit statistics for Alcohol% confirm the stability and accuracy of the model developed for maximization of ethanol yield based on sugar concentration, temperature, and recycled yeast inoculum. The Predicted R^2 value of 0.9264 confirms superior predictive ability to new data points, and Adjusted R^2 value of 0.9848 confirms an incredibly good fit with the current experimental data considering the number of parameters. That the gap between these two values is minimal (less than 0.2) means that the model is not over fitting and is stable. Secondly, a score of 28.052 for Adequate Precision a score much higher than the cut off score of 4 means a high signal to noise ratio in the sense that the model is very good at distinguishing real effects from noise. In short, all the above values assure that the model is stable, consistent, and optimal for process analysis and optimization of the fermentation process to obtain maximum alcohol.

4.3.2.1.2. Analysis of Data for Response 2: Residual Sugar% in Fermentation of Recycled Yeast Inoculum

Table 4.25: ANOVA for Residual Sugar% of Recycled Yeast Inoculum

Source	Sum of Squares	df	Mean Square	F-value	p-value	
Model	2.32	9	0.2576	87.96	< 0.0001	Significant
A-Sugar concentration	0.9781	1	0.9781	333.98	<	

					0.0001	
B-Temperature	0.2655	1	0.2655	90.64	< 0.0001	
C-Recycled yeast inoculum	0.0952	1	0.0952	32.50	0.0007	
AB	0.0000	1	0.0000	0.0000	1.0000	
AC	0.0025	1	0.0025	0.8537	0.3863	
BC	0.0625	1	0.0625	21.34	0.0024	
A^2	1.23	1	1.23	419.25	< 0.0001	
B^2	0.1520	1	0.1520	51.90	0.0002	
C^2	0.1946	1	0.1946	66.46	< 0.0001	
Residual	0.0205	7	0.0029			
Lack of Fit	0.0125	3	0.0042	2.08	0.2451	not significant
Pure Error	0.0080	4	0.0020			
Cor Total	2.34	16				

Table 4.25: ANOVA of Residual Sugar% tests the model to test the significance for predicting residual sugar content of the fermentation using the assistance of sugar concentration, temperature, and recycled yeast inoculum. Model F-value = 87.96 and p-value < 0.0001, which indicates that model and chosen variables are significant to describe residual sugar variation. Of the independent variables, sugar content (F = 333.98), temperature (F = 90.64), and recycled yeast inoculum (F = 32.50) are highly significant ($p < 0.001$), as anticipated, validating their key roles. Variables containing squared variables (A^2, B^2, and C^2) are also important, showing that there are nonlinear terms in the model. F-value for Lack of Fit is 2.08 and p-value 0.2451, which is not significant, which means experimental data fit exactly to the model and there is no significant lack of fit that is not accounted for by the model.

Table 4.26: Fit Statistics for Residual Sugar% of Recycled Yeast Inoculum

Std. Dev.	0.0541		R^2	0.9912
Mean	1.66		Adjusted R^2	0.9800
C.V. %	3.25		Predicted R^2	0.9091
			Adeq Precision	25.9003

Table 4.27: Residual Sugar% fit statistics validates the predictive ability and worth of the model in forecasting residual sugar after fermentation in terms of sugar concentration,

temperature, and recycled yeast inoculum. Predicted R^2 value of 0.9091 is an evidence of exceptional predictability power, whereas Adjusted R^2 value of 0.9800 is an evidence of exceptional fit to the experimental data. The tiny gap between these two figures (less than 0.2) indicates the model is stable and not over fitting. Moreover, the Adequate Precision value of 25.9003 is significantly higher than the threshold of 4, which is typical of a high signal to noise ratio.

4.4. Comparison of Ethanol %, Residual Sugar % and Improvement in overall efficiency with the Baseline Methods Presently Used to BALF.

The current fermentation process of Balezaf Alcohol and Liquor Factory (BALF) is yielding 6.5–9.5% v/v ethanol contents with 2.0–3.5% residual sugar content in the broth (Balf, 2024). High value residual sugar content signifies uncompleted sugar conversion, which reduces not only the ethanol yield but also contributes more organic material into the effluent. At the same time, the factory also generates huge quantities of fermentation sludge at 10–15% of the fermenter volume and this poses further inconvenience in waste treatment and downstream processing (Balf, 2024).

On the contrary, the optimized lab scale fermentation runs of the present work clearly demonstrated alcohol productivity improvement coupled with waste reduction. Ethanol concentration at maximum was 8.1–12.1% v/v, which is significantly higher than the factory baseline level. Moreover, residual sugar was also minimized to as low as 0.8–2.2%, which indicates much improved fermentable sugar utilization. Residual sugar prevention not only comes with benefits in enhanced ethanol production but also restricts the sludge formation, raising the efficiency of the process and downstream processing. It increases the demand of well balanced and total fermentation in reducing liquid and solid waste products in distilleries such as BALF. This comparison evidently shows that the optimized process of fermentation provides greater ethanol, lower wastage of sugar, and lower sludge compared to the existing process of BALF. These conditions if implemented at industrial scale would result in a high level of operating efficiency and an overall reduction in environmental concerns.

With the optimized conditions, the average ethanol concentration went up from 8.0% to 10.1% v/v, a rise of 26.25% above the factory benchmark. The average residual sugar at the same period went down from 2.75% to 1.5%, a reduction of 45.45%. Efficiency of the process was increased by approximately 35.85% (with improved sugar consumption, ethanol

production yield and reduced environmental stress). This strongly confirms that the improved process is extremely efficient and environment friendly than the conventional process in the factory.

Chapter Five

5. Conclusion and Recommendation

5.1. Conclusion

The yeast propagation and molasses fermentation process of Balezaf Alcohol and Liquor Factory (BALF) was optimized with successfully through Response Surface Methodology (RSM) and Box-Behnken Design (BBD). The optimal operating conditions of the yeast propagation determined at pH 4.75, 1.0 g/L DAP + urea, and 1.5 g/L dry yeast produced 0.8% alcohol and had the highest biomass value of yeast as 3.52×10^8 cells/mL. Maximum performance during fermentation occurred at 17% sugar concentration, 30°C, and 10% inoculum concentration, resulting in 12.1% (v/v) ethanol concentration and lowering residual sugar to 0.7%. Recycling of yeast was also effective, resulting in a maximum of 10.9% ethanol; hence it is viable for reuse. Statistical verification using ANOVA ($p < 0.05$) and $R^2 > 0.98$ validated the authenticity of the model. These gains significantly exceeded BALF's management (2–3.5% residual sugar, 6.5–9.5% yield for ethanol), and additionally resulted in significant in waste fermentation. Overall, the optimized process is a scalable, feasible, and environmentally friendly approach for augmenting ethanol productivity with less generation of waste in industrial molasses based bioethanol systems.

5.2. Recommendation

Application of optimized conditions of fermentation of 17% sugar, 30°C, 10% inoculum, and 1.0 g/L DAP + urea is recommended for increased yield of ethanol and minimized residual sugar. Application of molasses pretreatment is also recommended for improved sugar quality and fermentation efficiency. Yeast can be used twice for reuses to save money, and the important parameters need to be controlled continuously. The plant is also prompted to pursue scale up, continuous fermentation, and sludge biogas recovery for facilitating cleaner and more efficient production of ethanol. Follow up action can be aimed at scaling up nutrient species and performing long term industrial scale up in sustainable ethanol manufacturing.

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Appendices

Appendix A: Dilution of Molasses for 14 ^{0}Brix (8.05%), 24.34 ^{0}Brix (14%), 29.56 ^{0}Brix (17%) and 34.78 ^{0}Brix (20%) from 80 ^{0}Brix (46.02%) to Know Mass of Molasses Needed

A.1. Calculation for 14 ^{0}Brix in 10 L Yeast Propagation

Brix of molasses from Wonji sugar factory = 80 ^{0}Brix

Specific gravity (SG) of 80 ^{0}Brix = 1.4250

Specific gravity (SG) of 14 ^{0}Brix = 1.0568

$$\text{Mass of Molasses required in Kg} = \frac{\text{Brix needed} \times \text{SG} \times \text{Volume of sample preparation}}{\text{Original Brix of molasses} \times \text{SG of original Brix}}$$

$$\text{Mass of Molasses required in Kg} = \frac{14 \times \frac{1.0568}{\text{L}} \times 10\ L}{80 \times 1.4250}$$

Mass of Molasses required in Kg = 1.3 Kg

Therefore, the mass of molasses required to prepare 10 L yeast propagation is 1.3 Kg

A.2. Calculation for 24.34 ^{0}Brix in 1 L Molasses Fermentation

Brix of original molasses = 80 ^{0}Brix

Specific gravity (SG) of 80 ^{0}Brix = 1.4250

Specific gravity (SG) of 24.34 ^{0}Brix = 1.1026

$$\text{Mass of Molasses required in Kg} = \frac{\text{Brix needed} \times \text{SG} \times \text{Volume of sample preparation}}{\text{Original Brix of molasses} \times \text{SG of original Brix}}$$

$$\text{Mass of Molasses required in Kg} = \frac{24.34 \times \frac{1.1026}{\text{L}} \times 1\ L}{80 \times 1.4250}$$

Mass of Molasses required in Kg = 0.24 Kg

Therefore, the mass of molasses required to prepare 1 L molasses fermentation at 24.34 ^{0}Brix is 0.24 Kg

A.3. Calculation for 29.56 ^{0}Brix in 1 L Molasses Fermentation

Brix of original molasses = 80 ^{0}Brix

Specific gravity (SG) of 80 ^{0}Brix = 1.4250

Specific gravity (SG) of 29.56 ^{0}Brix = 1.1271

$$\text{Mass of Molasses required in Kg} = \frac{\text{Brix needed} \times \text{SG} \times \text{Volume of sample preparation}}{\text{Original Brix of molasses} \times \text{SG of original Brix}}$$

$$\text{Mass of Molasses required in Kg} = \frac{29.56 \times \frac{1.1271}{\text{L}} \times 1\ L}{80 \times 1.4250}$$

Mass of Molasses required in Kg = 0.29 Kg

Therefore, the mass of molasses required to prepare 1 L molasses fermentation at 29.56 ^{0}Brix is 0.29 Kg

A.4. Calculation for 34.78 ^{0}Brix in 1 L Molasses Fermentation

Brix of original molasses = 80 ^{0}Brix

Specific gravity (SG) of 80 ^{0}Brix = 1.4250

Specific gravity (SG) of 34.78 ^{0}Brix = 1.1525

$$\text{Mass of Molasses required in Kg} = \frac{\text{Brix needed} \times \text{SG} \times \text{Volume of sample preparation}}{\text{Original Brix of molasses} \times \text{SG of original Brix}}$$

$$\text{Mass of Molasses required in Kg} = \frac{34.78 \times \frac{1.1525}{\text{L}} \times 1\ L}{80 \times 1.4250}$$

Mass of Molasses required in Kg = 0.35 Kg

Therefore, the mass of molasses required to prepare 1 L molasses fermentation at 34.78 ^{0}Brix is 0.35 Kg

Appendix B: Actual and Predicted values of Yeast Propagation for Nutrients (DAP+urea)

B.1. Actual and Predicted Values for Response 1: Alcohol %

Table B.1: Actual and predicted values alcohol %

Run Ord	Actual	Predicted	Residual	Leverage	Internally	Externally	Cook's Distan	Influence on	Standard

er	Valu e	Value			Studentiz ed Residual s	Studentiz ed Residual s	ce	Fitted Value DFFIT S	Order
1	1.00 00	0.9200	0.0800	0.200	1.256	1.321	0.039	0.661	17
2	0.90 00	0.9200	- 0.0200	0.200	-0.314	-0.293	0.002	-0.146	6
3	0.90 00	0.9200	- 0.0200	0.200	-0.314	-0.293	0.002	-0.146	1
4	1.00 00	0.9200	0.0800	0.200	1.256	1.321	0.039	0.661	12
5	0.80 00	0.9200	- 0.1200	0.200	-1.884	-2.484	0.089	-1.242	2
6	2.20	2.19	0.0125	0.750	0.351	0.328	0.037	0.568	9
7	2.10	2.06	0.0375	0.750	1.053	1.063	0.333	1.841	8
8	1.90	1.87	0.0250	0.750	0.702	0.674	0.148	1.168	4
9	1.60	1.60	0.0000	0.750	0.000	0.000	0.000	0.000	14
10	2.10	2.14	- 0.0375	0.750	-1.053	-1.063	0.333	-1.841	5
11	1.70	1.71	- 0.0125	0.750	-0.351	-0.328	0.037	-0.568	11
12	2.10	2.10	0.0000	0.750	0.000	0.000	0.000	0.000	13
13	1.80	1.82	- 0.0250	0.750	-0.702	-0.674	0.148	-1.168	10
14	2.00	2.01	- 0.0125	0.750	-0.351	-0.328	0.037	-0.568	7
15	1.80	1.84	- 0.0375	0.750	-1.053	-1.063	0.333	-1.841	15
16	1.60	1.59	0.0125	0.750	0.351	0.328	0.037	0.568	3
17	1.90	1.86	0.0375	0.750	1.053	1.063	0.333	1.841	16

Table B.2: Actual and predicted value of yeast cell count

Run Ord er	Actual Value	Predicte d Value	Residua l	Lever age	Internall y Studenti zed	External ly Studenti zed	Cook' s Dista nce	Influe nce on Fitted Value	Stand ard Order

					Residuals	Residuals		DFFITS	
1	3.430E+08	3.474E+08	-4.400E+06	0.200	-1.150	-1.182	0.033	-0.591	17
2	3.500E+08	3.474E+08	2.600E+06	0.200	0.679	0.651	0.012	0.325	6
3	3.480E+08	3.474E+08	6.000E+05	0.200	0.157	0.145	0.001	0.073	1
4	3.440E+08	3.474E+08	-3.400E+06	0.200	-0.888	-0.873	0.020	-0.437	12
5	3.520E+08	3.474E+08	4.600E+06	0.200	1.202	1.249	0.036	0.624	2
6	2.530E+08	2.525E+08	5.000E+05	0.750	0.234	0.217	0.016	0.376	9
7	2.700E+08	2.730E+08	-3.000E+06	0.750	-1.402	-1.531	0.590	-2.651(1)	8
8	2.720E+08	2.758E+08	-3.750E+06	0.750	-1.753	-2.166	0.921	-3.751(1)	4
9	3.080E+08	3.083E+08	-2.500E+05	0.750	-0.117	-0.108	0.004	-0.188	14
10	2.680E+08	2.650E+08	3.000E+06	0.750	1.402	1.531	0.590	2.651(1)	5
11	2.880E+08	2.885E+08	-5.000E+05	0.750	-0.234	-0.217	0.016	-0.376	11
12	2.670E+08	2.668E+08	2.500E+05	0.750	0.117	0.108	0.004	0.188	13
13	2.820E+08	2.783E+08	3.750E+06	0.750	1.753	2.166	0.921	3.751(1)	10
14	2.710E+08	2.718E+08	-7.500E+05	0.750	-0.351	-0.327	0.037	-0.567	7
15	2.900E+08	2.868E+08	3.250E+06	0.750	1.519	1.717	0.692	2.975(1)	15
16	3.060E+08	3.053E+08	7.500E+05	0.750	0.351	0.327	0.037	0.567	3
17	2.780E+08	2.813E+08	-3.250E	0.750	-1.519	-1.717	0.692	-2.975(1	16

			+06					)	

Appendix C: Comparison of Ethanol %, Effluent Production in Terms of Residual Sugar % and Improvement in overall efficiency with the Baseline Methods Presently Used to BALF

C.1. Summary table of comparison

Table C.1: Summary of comparison

Responses	BALF Average	Optimized Average	Change
Ethanol %	(6.5+9.5)/2 = 8	(8.1+12.1)/2 = 10.1	26.25 %
Residual Sugar %	(2+3.5)/2 = 2.75	(0.8+2.2)/2 = 1.5	45.45 %

C.2. Overall efficiency improvement calculation

We improve efficiency based on:

- Ethanol production multiplier (expressed as % increase in ethanol production)
- Reduction in residual sugar (%)

Therefore, the total efficiency improvement in the process is given by:

Total Efficiency $= \frac{26.26+45.45}{2} = 35.85$