STUDIES ON BIOETHANOL PRODUCTION FROM RICE STRAW BY *Saccharomyces cerevisiae*

by

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A Thesis submitted to

Faculty of Basic Sciences

in partial fulfilment of the requirements

for the degree of

MASTER OF SCIENCE

IN

MICROBIOLOGY

Division of Microbiology, Faculty of Basic Sciences

Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu,

Main Campus, Chatha, Jammu – 180009

2022

CERTIFICATE-I

This is to certify that the thesis entitled **"Studies on bioethanol production from rice straw by *Saccharomyces cerevisiae*"** submitted in partial fulfilment of the requirements for the degree of **Master of Science in Basic Sciences (Microbiology)**, to the Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu, is original work and has similarities with published work not more than minor similarities as per UGC norms of 2018 adopted by the university. Further the level of minor similarities has been declared after checking the manuscript with **Urkund** software provided by the University.

This work has been carried out by **Miss. Neeru Choudhary**, under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma. It is further certified that help and assistance received during the course of thesis investigation have been duly acknowledged.

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Acknowledgement

Acknowledgement

First and the foremost, I express my heartiest devotion to God Almighty my creator, my strong pillar, my source of inspiration, wisdom, knowledge and understanding. He has been the source of my strength throughout this program and on His wings only have I soared.

I feel great pride and privilege to express my profound gratitude and heartiest indebtedness and obligation to my esteemed Major Advisor, **Dr. Brojeshwar Singh**, Assistant Professor, Faculty of Basic Sciences, Sher-e-Kashmir University of Agricultural Sciences and Technology-Jammu, for his inspiring and ingenious guidance, articulate criticism, cordial discussion, unending zeal, and constant encouragement, which helped me to overcrowd the competition. I will be eternally grateful for his wisdom, kindness, concern and her encouragement to write this thesis made me confident that the topic was worthy of my investigation—and that my investigation was worthy of the topic

I would like to express my special thanks of gratitude to the worthy members of my advisory committee **Dr. Upma Dutta** (Assistant Professor, Microbiology), **Dr. A.K, Singh** (Associate Professor, Biotechnology) and Dean's Nominee, **Dr. Sanjay Guleria** (Professor and head, Biochemistry) for their intellectual enlightenment and pertinent suggestions throughout the pursuit of this study.

I am grateful for the assistance provided by **Dr. S.E.H.Rizvi** (Head of Division, Microbiology) and for his knowledgeable and mature guidance and ever keen assistance in bringing the work to a successful conclusion.

I shall fail in my duty, if I don't Thank the non- teaching staff of my department- **Sh. Ajeet Ram ji, Mr. Raman Chaudhary**, for their help and assistance during the present study.

Moments of delight and grief shared with my dear colleagues, seniors and juniors, **Sapalika**, **Khalid**, **Somal**, **Shruti**, **Boomika**,**Yasmin**, **Harkiran**, and **Vironika**, for their prompt assistance, best wishes, and lively company, who has shared the movements of laughter and sorrow across miles, can never be imagined.

The officials of the government and non- government of and other concerned authorities are also thankful for their keen interest and great

support while conducting the study. Distinguished personalities, the locals and the others stakeholders contacted, interviewed and the source of information revealed are also thankful acknowledged.

I must also thank my parents and friends for the immense support and help during the research work. Without their help, completing this research work would have been very difficult.

Finally, I am extremely grateful for my parents whose constant love and support keep me motivated and confident. My accomplishments and success are because they believed in me. Deepest thanks to my siblings, who keep me grounded, remind me of what is important in life, and are always supportive of my adventures.

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Date: 14/02/22

ABSTRACT

Title of Thesis	**: "Studies on bioethanol production from rice straw by *Saccharomyces cerevisiae*"**
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Registration No.	**: J-19-MBS-38**
Major subject	**: Microbiology**
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Degree to be awarded	**: M.Sc. (Microbiology)**
Year of award of degree	**: 2022**
Name of university	**: Sher-e-Kashmir University of Agricultural Sciences & Technology of Jammu**

ABSTRACT

An investigation entitled 'Studies on bioethanol production from rice straw by *Saccharomyces cerevisiae*' was conducted in the Division of Microbiology, Sher-e-Kashmir University of Agricultural Sciences,with a view to study the bioethanol producing potential of locally isolated *Saccharomyces cerevisiae*strain;using Rice straw as a substrate.Optimization of fermentation parameters i.e., temperature, pH and agitation to enhance the bioethanol production was also studied. Fifteen yeast strains were isolated from fruit sample (Apple, Papaya, Sugarcane, Grapes, Orange, Pineapple) and Kaladi samples collected from local markets of Jammu and Udhampur district respectively. Potential yeast isolates were identified on the basis of morphological and microscopic characteristics which were similar to that of MTCC 170 culture procured from IMTECH-Chandigarh. Out of 15 isolates, 14 show a positive change in the color of screening medium indicating the production of ethanol. S-9 culture was further selected based on its highest bioethanol producing ability of 17.88 mg/mL at 24 h of fermentation on YEPD medium and hence chosen for the fermentation of rice straw hydrolysate.Rice straw was subjected to alkaline and acidic pretreatment which was followed by enzymatic hydrolysis.Ethanol Fermentation of the total Rice Straw Hydrolysate obtained after pretreatments using S-9 culture revealed maximum ethanol concentration of 12.26 mg/mL at 24h havingfermentation efficiency of 69.5%, 50.7% of sugar conversion occur during the fermentation process and volumetric productivity of 0.1702 g/L/h. Ethanol content per gram of sugar utilized was estimated to be 0.168g/g. Further, S-9 culture show the maximum efficiency for bioethanol production on YEPD medium at temperature of 30°C, pH of 5.6 and under static conditions.

Key words: Fermentation, *Saccharomyces cerevisiae*, Bioethanol, Rice straw, Fermentation Efficiency.

Signature of Major Advisor

Neeru Choudhary

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LIST OF ABBREVATION

LB	Lignocellulosic biomass	**mg g^{-1}**	Milligram per gram
SSF	Solid state fermentation	**%**	Percent
TRL	Technology readiness level	**g/L**	Gram per liter
ºC	Degree Celsius	**ABE**	Acetone–butanol–ethanol fermentation
CS	Cotton stack	**SCB**	Sugarcane bagasse
IL	Ionic liquid	**PEG**	Polyethylene glycol
FTIR	Fourier transform infrared spectroscopy	**H_2S**	Hydrogen sulphide
XRD	**X**-ray diffraction	**Zym**	*Zymomonas mobilis*
SEM	Scanning electron microscopy	**wt.**	Weight
DSAP	Dilute sulfuric acid pretreatment	**v/v**	Volume by volume
UAAP	Ultrasound assisted alkali pretreatment	**g/L/h**	Gram per liter per hour
HPAAP	High pressure-assisted alkali pretreatment	**g/kg**	Gram per kilogram
MJ/kg	Mega joule per kilogram	**g/g**	Gram per gram
OPCB	One pot consolidated bioprocess	**STW**	Spent tea waste
PNB	Pine needle biomass	**g EtOH/L**	Gram ethanol per liter
Reducing sugar/L	Reducing sugar per liter	**LPCM**	Lignin plastic composite material
UV-C	Ultra violet C	**w/w**	Weight by weight
RFLP	Restricted fragment length polymerization	**w/v**	Weight by volume
CNG	Compressed natural gas	**AFEX**	Ammonia fibre explosion
GHG	Greenhouse gases	**1st**	first
SE	Steam explosion	**2nd**	second
DA	Dilute Sulphuric acid Hydrolysis	**mL**	milliliters
BOD	Biological oxygen demand	**psi**	Pound per square inch
@	At the rate	**min.**	minute
mg/mL	Milligram per milliliter	**h**	hour
i.e.,	That is	**YEPD**	Yeast extract peptone dextrose

g	gram	**No.**	Number
et al	et alia = and other	**pH**	Power of hydrogen
Fig.	Figure	**mg**	milligram
S.No.	Serial number	**YMA**	Yeast mannitol agar

Chapter-1

Introduction

CHAPTER-1

INTRODUCTION

Throughout the world, fossil fuels are the primary source of energy. Due to its non-renewable nature and increased carbon dioxide emission, it is a major cause of global warming. The depriving fossil fuel resources has forced mankind to depend on an alternative fuel which must be renewable, and bioethanol is one of them. Bioethanol is considered as an important renewable fuel to partly replace fossil derived fuels. Fuel or energy sources that are obtained from organic by-products or naturally occurring, living organisms are called as Biofuels. Paper and wood waste, agrowaste, kitchen waste are major sources of biofuels. Ethanol or Ethyl alcohol is an oxygen containing organic compound having the chemical formula -C_2H_5OH. It is a monohydric primary alcohol which boils at 78.5 **ºC** (Rahman, 2013). Ethanol is used as a solvent, a germicide, a beverage, an antifreeze, a fuel and as a chemical intermediate for organic chemicals. It is a colorless, clear, flammable and slightly toxic chemical with acceptable odour. It can be produced from petrochemical feedstock by acid-catalyzed hydration of ethene which account for only 3-4% of the total production while the rest is produced from biomass feedstock through fermentation (Licht, 2006). A blend of 10% dry ethanol and unleaded gasoline was commercially introduced into the US and continues to be marketed mainly in the Midwestern States (Hansen *et al.*, 2005). The octane number and combustion efficiency of bioethanol is greater than gasoline. It has high compression ratio, reduced burning time and high heat of vapourisation (Carrillo-Nieves *et al*., 2019, Balat *et al*., 2008). After combustion, ethanol emits less quantity of particulate matters, hydrocarbons and NO_x because of the oxygen content i.e., 35% oxygen therefore it's emission and toxicity are relatively less than other fuels such as diesel, petroleum (Toor *et al*., 2020). Increase in bioethanol from 50 million m^3 in 2007 to over 100 million m^3 in 2012 across the world. Brazil and United States contribute 80% of the world supply, mostly using corn or sugarcane (Kang *et al.*, 2014). Fermentation is the process of chemical changes by which breakdown of larger molecules into smaller molecules occur by the microbial intervention. Alcoholic fermentation is a widely used process for production of Bioethanol. Ethanol fermentation is a biochemical process by which breakdown of sugars such as glucose, fructose, and sucrose occur in order to yield energy and gives ethanol and carbon dioxide as biproducts. Yeasts are unicellular eukaryotic

microorganisms which belongs to kingdom fungi. About 80% of ethanol is produced by anaerobic fermentation, caused by yeast belongs to *Saccharomyces cerevisiae* (Nofemele *et al.*, 2012). It is widely distributed in natural habitat and classified under division Ascomycota. They are heterotrophic, require water for diffusion and can grow aerobically as well as anaerobically.

Ethanol fermentation is usually carried out using sugars, starch and cellulose as substrate. First generation bioethanol includes the production of ethanol from lignocellulose material as feedstock, second generation include sugar based raw material and third generation include algal bioethanol production (Kang *et al.*, 2014). Sugar and starch are currently the main raw material but due to their demand for human feed and an expensive feedstock in the near future, this would guide the attention towards the cellulosic matter as the only potential feedstock for the production of ethanol (Taherzadel *et al.*, 2007).

Agricultural or forestry residues, municipal garbage, and energy crops are all examples of lignocellulosic biomass. It is a plentiful, economical, and renewable feedstock with disposal issues. LB is an intrinsically complex and recalcitrant material in which cellulose, hemicellulose, and lignin are tightly linked, making it difficult for hydrolyzing enzymes to access. (Pin *et al.*, 2019; Trinh *et al.*, 2018).

As a result, a pretreatment method is an absolute requirement for achieving a cost-effective reduction in the downstream operation costs of fermentable sugars recovery from biomass (Tomas-Pejo *et al.*, 2008, Curreli *et al.*, 2002). For acid treatments, (a) high temperature and low acid concentration, while (b) low temperature and high acid concentration are being used. H_2SO_4 is often used in diluted form for a wide range of biomasses, including switch grass, maize stover, spruce, poplar, kans grass, coconut fibre, and rice/wheat straw (Zhu *et al.*, 2008). The production of several microbial growth inhibitors such as acetic acid, furfural, and 5-hydroxymethyalfurfural, that must be detoxified before fermentation, is the major disadvantage of acid treatment (Palmqvist and Hahn-Hägerdal, 2000). In general, a higher pretreatment temperature and a shorter residence period result in better xylose recovery and enzymatic digestibility. All existing investigations in the same research field used diluted H_2SO_4 concentration of (0.2–2%) and higher temperature (121–372°C) to promote the most critical functions such as hemicellulose hydrolysis,

cellulose exposure for digestion, and heavy metal solubilization (Balat, 2011). Rice straw can be utilized to make bio-ethanol, providing additional revenue and a long-term use. It would also provide a clean energy answer to India's ever-growing energy demand. However, it is becoming increasingly crucial to investigate the long-term viability of bio-ethanol production from rice straw, as well as how it fits into the current Indian agricultural landscape (Singh *et al.*, 2002).

In the light of above facts, the present study aims to produce bioethanol using rice straw as a substrate from the isolated strain of *Saccharomyces cerevisiae* with the following objectives;

1. Isolation of bioethanol producers from fruit and dairy sources.
2. Optimization of bioethanol yield and quality parameters.

Chapter-2

Review of Literature

CHAPTER 2

REVIEW OF LITERATURE

Fuel ethanol derived from biomass appears to be a promising alternative to traditional fossil fuels, having the potential to be used as a sole fuel in specialized engines or in fuel mixes. In the present study, we aim to produce bioethanol using rice straw as a raw material. And to find out the ability of wild strain of *Saccharomyces cerevisiae* to ferment glucose efficiently, so as to ensure a cheap source of raw material for the production of bioethanol.

Substrate for bioethanol production:

In recent decades, ethanol made from renewable resources has piqued interest as a potential replacement for fossil fuels. Because of the rising reliance on oil and conventional fuels in recent years, its production has become much more important. Lignocellulosic biomass, can be found in the form of grass, wood, and crop leftovers, provides a renewable and greenhouse-gas-friendly source of sugars that can be fermented to ethanol. It is widely acknowledged that lignocellulosic materials have the potential to be used in the production of bioethanol (Ruchi *et al.*, 2011). Straws, one of the abundant agricultural by-products, can supply appropriate feedstocks for competitive energy production, reducing reliance on fossil fuels (Ibrahim, 2012). Straw is a promising material for the generation of bioethanol fuel because of its high cellulose and hemicellulose content, which can be easily hydrolyzed into fermentable sugars, and other characteristics (Ibrahim, 2012). Because of the high energy content of cellulose, lignocellulosic materials comprising cellulose, hemicellulose, and lignin are potential bioethanol conversion substrates. Despite the fact that lignocellulosic biomass stores energy in the form of cellulose and hemicellulose, lignin acts as a barrier to their hydrolysis. Cellulolytic enzymes hydrolyze cellulose and hemicelluloses to release fermentable sugars after a dilute acid pretreatment disintegrates the biomass complex (Nanda *et al.*, 2014). Pretreatment of lignocellulosic biomass causes significant lignocellulosic structural breakdown, fractional hydrolysis of hemicellulosic content, lignin component depolymerization, and defibration. Acid, alkali, steam explosion, and ozonolysis are some of the pretreatment procedures followed (Mtui, 2009). Due to the cost/energy intensive characteristics of lignocellulosic biomass (LB) pretreatment and

saccharification, lignocellulosic-bioethanol-fuel technology faces problems. Pretreatment of LB with ionic liquid (IL) has recently developed as an environmentally acceptable method (Vaid *et al.*, 2018)

In the recent years Toor *et al.*, (2020) found that Pretreatment, effective sugar release, and fermentation are all key obstacles to commercialization of lignocellulosic ethanol. Several techniques, such as simultaneous saccharification and fermentation, have been developed to address these issues (SSF). In his research paper, he discussed about the pros and disadvantages of various technologies involved in ethanol manufacturing. Single-pot biorefineries, combined bioprocessing, and bioenergy systems with carbon capture are all promising new technologies. These technologies, on the other hand, have a lower technology readiness level (TRL), suggesting that more work is required before they can be considered for commercial availability. Solving energy demands is not solely a technological problem, and the interdependence of numerous aspects must be considered in addition to technological advancements.

Binod *et al.*, (2010) found that as rice straw is one of the most abundant renewable resources, it is an appealing lignocellulosic material for bioethanol production. It has multiple aspects, including a high amount of cellulose and hemicelluloses that can easily be converted into fermentable sugars. He researches that, there are various difficulties and restrictions in the conversion of rice straw to ethanol. Rice straw is an inferior feedstock for ethanol production due to its high ash and silica content. The selection of an effective pretreatment process is one of the primary issues in creating technology for bioethanol production from rice straw The selection of pretreatment procedures is critical for increasing the efficiency of enzymatic saccharification and making the entire process economically viable.

Belal (2013) worked over the production of bioethanol from rice straw residues. From decayed rice straw leftovers, a cellulose-utilizing mould was isolated. *Trichoderma reesei* was identified as the efficient rice straw degrading microorganism. Different carbon sources in liquid culture, such as rice straw, carboxymethyl cellulose, filter paper, sugar cane bagasse, cotton stalk, and banana stalk, stimulated cellulase production in. *T. reesei*, whereas glucose or Potato Dextrose suppressed cellulase synthesis. The solid-state culture of *T. reesei* on rice straw medium produced cellulase. The ideal temperature and pH at which *T. reesei* cellulase was produced were 6ºC and 25ºC, respectively. Rice straw had varying susceptibilities to cellulase when it came to

converting to reducing sugars. He found that the overall trend of rice straw bioconversion with cellulase was higher than that of *T. reesei*. This enzyme changed acid, alkali, and ultrasonic processed cellulose from rice straw to glucose, which was subsequently fermented to produce ethanol. After 7 days of fermentation with *S. Cerevisiae*, the combined method of acid pretreatment with ultrasonic and subsequent enzyme treatment resulted in the maximum conversion of lignocellulose in rice straw to sugar and, as a result, the highest ethanol concentration. The ethanol yield was between 10 and 11 g/L.

Li *et al.*, (2016) worked over the effect of acid pretreatment over the various parts of corn stalk. They analysed the impacts of several portions of the corn stalk, such as the stem, leaf, blossom, cob, and husk, on second-generation ethanol production. The effect of dilute acid pretreatment was investigated using FTIR, XRD, and SEM. After pretreatment, the bagasse was further hydrolyzed by cellulase and employed as the ethanol fermentation substrate. They found that, cob had a higher sugar content and better enzymatic digestion than other components of the corn stalk. When corn was employed as a feedstock, the highest glucose yield of 94.2 percent and ethanol production of 24.0 g/L were obtained, but the glucose yield and ethanol production of flower were only 86 percent and 17.1 g/L, respectively.

Wang *et al.*, (2016) studied about the production of bioethanol from cotton stalk. They found cotton stalk (CS) as a promising biomass for bioethanol production, but due to the resistant nature of lignocellulose, direct conversion without pretreatment always yields an incredibly poor yield. The impacts of different approaches, such as dilute sulfuric acid pretreatment (DSAP), ultrasound assisted alkali pretreatment (UAAP), and high pressure-assisted alkali pretreatment (HPAAP), were investigated. The complete structures of pretreatment CS were clearly disturbed, according to the research. After pretreatments, the hemicellulose and lignin in biomass were eliminated, and the crystallinity of cellulose was raised. In comparison to UAAP and DSAP, HPAP produced the highest reducing sugar and ethanol yields (271.70 mgg^{-1} and 45.53 % respectively). HPAP has been shown to be a viable and successful pretreatment method for ethyl alcohol.

Bioethanol

Ethanol is one of the most important clean fuels and renewable energy resources, and it could play a key part in resolving the looming oil storage

dilemma (Rogers *et al.*, 2007). Ethanol is utilized as a fuel because it has several advantages, including a lower thermal energy content (approximately 45 percent less per gallon than diesel), a low price, and lower emissions than diesel.

Because ethanol has a higher-octane number (99) than gasoline (80–100), preignition does not occur when it is used. As a result, it is widely utilized as a competitive fuel addition with gasoline, however it is rarely used in its pure form (Oliveira *et al.*, 2005). Ethanol is being studied extensively as a renewable fuel source since it is equivalent to gasoline in many ways (Nasir *et al.*,2017). It is the most promising liquid fuel since it can be manufactured easily from a variety of agriculturally based renewable components (Wigmosta *et al.*, 2011). Bioethanol is the most widely utilized biofuel in the world, as it helps to reduce crude oil use and pollution. Sucrose, starch, and other feedstocks can all be used to make it. It can be made from a variety of feedstocks, including sucrose, starch, lignocellulosic and algal biomass, using a microorganism-mediated fermentation process (Azhar *et al.*, 2017).

Sumbhate *et al.*, (2012) investigated the direct reaction approach for measuring ethanol in alcoholic beverages. For detecting total ethanol in marketed alcoholic-drink brands, a simple and sensitive colorimetric method using a 4 percent solution of sodium dichromate, sulfuric acid, and an acetate buffer pH 4.3 was devised. A color reaction of ethanol with sodium dichromate is used in this approach. The colorimetric measurement was based on the creation of green colored chromate ions after treating ethanol with sodium dichromate as the limiting reactant in the presence of sulfuric acid and an acetate buffer pH 4.3. The maximum absorption of ethanol was found to be 578 nm. 0.6 mg/mL and 1.9 mg/mL were found to be the detection and quantification limits for ethanol, respectively. Despite the fact that the assay was only established for alcoholic beverages, it can easily be extended for the examination of other alcohol-containing medicinal preparations, beverages, and herbal formulations. Furthermore, the procedures outlined do not necessitate the use of costly equipment, chemicals, or talents, allowing researchers, alcohol processors, and beverage producers to benefit from them.

Nanda *et al.*, (2014) worked over the production of ethanol and butanol from lignocellulosic feedstock. The potential for lignocellulosic feedstock to sustain the renewable generation of biofuels like ethanol and butanol is enormous. They examined the effects of various H_2SO_4 doses (0–2.5%) on three lignocellulosic feedstock materials, namely pinewood, timothy grass, and wheat straw, at 121°C for 1 hour. In

addition, the pretreated feedstock was enzymatically hydrolyzed at 45°C for 72 hours with cellulase, b-glucosidase, and xylanase. For ethanol and butanol production, biomass hydrolysates containing monomeric sugars (glucose and xylose) were fermented by *Saccharomyces cerevisiae* and *Clostridium beijerinckii,* respectively. From biomass hydrolysates, concentrations of ethanol and butanol, residual sugars, and byproducts like acetone, acetate, and butyrate were compared. The presence of 38.8, 34.2, and 39.1 wt. percent cellulose, 23.6, 30.1, and 24.1 wt. percent hemicellulose, and 20.4, 18.1, and 16.3 wt. percent lignin were found in pinewood, timothy grass, and wheat straw, respectively. The overall (water, ethanol, and hexane-soluble) extractives in biomass samples, on the other hand, were in the range of 15.7–19.2 wt. percent. Using 2 percent H_2SO_4 and enzymatic hydrolysis, the highest quantities of glucose (32.9 g/L) and xylose (35.6 g/L) were recovered from pinewood. From timothy grass and wheat straw hydrolysates, 1.5 percent H_2SO_4 pretreatment with enzymatic hydrolysis yielded 26.7 and 26 g/L glucose and 30.7 and 37.6 g/L xylose, respectively. Pinewood had the highest percentage of saccharification (29.6%), followed by timothy grass (24%) and wheat straw (23.4%). In 36 hours of fermentation, S. *cerevisiae* ATCC 96581 produced maximum ethanol concentrations of 20.3, 32.9, and 48.3 g/L from 50, 100, and 150 g/L glucose levels, respectively. Pinewood had the highest ethanol levels (24.1 g/L) among the feedstock hydrolysates, followed by wheat straw (23.2 g/L) and timothy grass (22.6 g/L). In 60 hours of ABE fermentation using C. *beijerinckii* B-592, the highest butanol concentrations were 11.2, 11.9, and 9.3 g/L from 50, 100, and 150 g/L glucose substrate. Pinewood (11.6 g/L) > Wheat straw (11.2 g/L) > Timothy grass (10.8 g/L) were the biomass hydrolysates with the lowest butanol contents. Additionally, after 60 hours of fermentation, total ABE levels from pinewood, timothy grass, and wheat straw hydrolysates were determined to be 18.5, 17.4, and 17.9 g/L, respectively.

Sharma *et al.*, (2019) used enzymes from an ionic liquid tolerant *Aspergillus assiutensis* VS34 to enhance saccharification utilizing ultrasonic and surfactant assisted ionic liquid pretreatment of sugarcane bagasse. In a biorefinery, ionic liquid (IL) pretreatment is an effective approach for fractionating lignocellulosic biomass (LB) to fermentable sugars. When sugarcane bagasse (SCB) was pretreated with IL (1-butyl-3-methylimidazolium chloride [Bmim]Cl) as well as a surfactant (PEG-8000), the sugar yield increased by 16.5 percent during enzymatic saccharification.

Mohseni *et al.*, (2015) study on isolating ethanol-producing bacteria, as well as characterizing, optimizing, and evaluating their ethanol production. Samples from diverse fruits, plant saps, and soils were screened for ethanol-producing bacteria, and the highest ethanol producer was determined. Six of the 37 ethanol-producing isolates were chosen for characterisation out of a total of 37. The pH, temperature, agitation, time, and beginning glucose concentration were all used to optimise bacterial growth and ethanol production. The majority of isolates came in pairs or in singles. All of the isolates were motile and catalase positive, however they were unable to hydrolyze gelatin and create hydrogen sulphide (H_2S). With an optimal pH of 6 and a growth temperature of 35°C, Zym6 produced the highest ethanol yield of 6.28 g/L. Furthermore, with xylose and tryptophan, Zym5 and Zym6 had the greatest ethanol yields of 19.52 g/L and 18.75 g/L, respectively. As a result, the best medium for ethanol synthesis was one with a pH of 6, a growth temperature of 35°C for 24-48 hours, and carbon and nitrogen sources of xylose and tryptophan.

Balakumar *et al.*, (2001) found that in addition, with xylose and tryptophan, Zym5 and Zym6 had the greatest ethanol yields of 19.52 g/L and 18.75 g/L, respectively. As a result, the best conditions for ethanol generation were a medium with a pH of 6, a growth temperature of 35°C for 24-48 hours, and carbon and nitrogen sources of xylose and tryptophan. Three stains p1, p2, and p3, were chosen from the distillery environment. These were exposed to 15 temperature treatment cycles, yielding three thermally adapted stains, pt1, pt2, and pt3, with viability of 100, 30, and 20% at 50ºCfor 30 minutes, respectively. When the incubation temperature was reduced to 40ºC, the duration of 100% viability increased from 24 hours (p1) to 68 hours (pt1). The selected strain was designated as *Saccharomyces cerevisiae* SI. *Saccharomyces cerevisiae* SI produced 46g/L (36h), 38g/L (48h), and 26g/L (48h) of ethanol from 100g glucose/L at 40, 43, and 45ºC, respectively.

Thammasittirong *et al.*, (2013) employed random UV-C mutagenesis to improve ethanol tolerance in *Saccharomyces cerevisiae* NR1 in order to increase ethanol production. UVNR56, an ethanol-tolerant mutant, demonstrated substantially better ethanol tolerance in the presence of 15% (v/v) ethanol and significantly higher viability during ethanol fermentation from sugarcane molasses and sugarcane molasses with initial high ethanol supplementation. UVNR56 generated a maximum ethanol concentration of 10.3 percent (v/v), productivity of 1.7 g/L/h, and theoretical yield of

98.7 percent from molasses medium at 37°C, whereas the wild-type produced 8.6 percent (v/v), 1.4 g/L/h, and 83.3 percent, respectively. Furthermore, during molasses fermentation with a 5 percent (v/v) ethanol initial supplement, the maximal ethanol concentration and productivity of UVNR56 were 25.7 percent and 42.9 percent higher, respectively, than the wild-type.

Hoşgün *et al.*, (2017) investigated the Hazelnut shell cellulose conversion to fermentable sugars and ethanol using low-temperature sodium hydroxide (NaOH) pretreatment, enzymatic saccharification, and ethanol fermentation. With 6 percent NaOH for 72 hours and a 1/10 solid/liquid ratio, maximum glucose recovery was achieved (48.33 g/100 g cellulose). 41.18% of the lignin was removed under these circumstances. The theoretical ethanol yield (ethanol produced/potential glucose in biomass) was 40.71 percent, the overall process efficiency was 37.73 g/kg biomass, the ethanol productivity was 0.115 g/L/h, and the fermentation efficiency was 96.7 percent. The low-temperature–long-residence-time alkali procedure used 34.8 MJ/kg untreated hazelnut shells in total.

Vaid *et al.*, (2018) studied the Pine Needle Biomass Consolidated Bioprocessing for Biofuel-Ethanol Production. Ionic liquid (IL) stable enzymes must be used to saccharify Ionic liquid (IL) pretreatment Lignocellulosic biomass (LB), or the latter may be inhibited. Furthermore, the availability of IL stable enzymes may aid in the development of novel consolidated processes by connecting IL-mediated LB pretreatment and enzymatic saccharification processes in a single vessel, i.e., one pot consolidated bioprocess (OPCB). For the first time, an OPCB was created and improved for bioethanol production from pine needle biomass (PNB) in a single pot employing in situ IL pretreatment and enzymatic saccharification. The sugar yield was 1.88 g per five gramme of PNB after optimising several process parameters such as biomass loading, reaction duration, and cellulase/xylanase enzyme dose. The sugar hydrolysate was fermented with a dual yeast culture, *Saccharomyces cerevisiae* and *Pichia stipitis*, to produce ethanol. After 72 hours of fermentation, a maximum ethanol output of 0.148 g/g PNB was recorded, resulting in a 41.39 percent efficiency. The research suggests that consolidated PNB processing could be a cost-effective, long-term, and practical method of valorizing PNB for industrial production of second-generation ethanol-biofuels.

Curreli *et al.*, (2002) researched enzymatic hydrolysis of pretreated wheat straw in an efficient manner. A two-step chemical pretreatment was proposed for fractionation of wheat straw components. Dilute Sulphuric acid hydrolyzed hemicelluloses, allowing for a significant recovery of crystalline xylose. Lignin was extracted using a mild alkaline/oxidative solubilisation method that did not use sulphite or chlorine or its derivatives. It was both inexpensive and environmentally beneficial to utilise diluted reagents and low temperatures. The pretreatment material was almost pure cellulose, and the enzymatic hydrolysis proceeded quickly and with high yields, yielding high glucose syrups of outstanding purity.

In conclusion, clear progress with the combined subsequent pretreatments proposed here has been achieved. Pure xylose yields were produced in reasonable quantities. The only component requiring a disposal treatment was a moderately alkaline waste from which more than 80% of lignin-derived phenolics had been oxidatively eliminated. This garbage, on the other hand, was significantly less hazardous to the environment than normal pulp-industry waste.

Saccharomyces cerevisiae

Yeasts are eukaryote fungus with only one cell. They're crucial in the manufacturing of traditional foods like bread, beer, and wine. They also aid in the production of a desirable flavor during the ripening of cheese (Boudjema *et al.*, 2016). Natural substrates such as leaves, flowers, sweet fruits, grains, tree exudates, insects, dung, and dirt are used to isolate yeasts (Boudjema *et al.*, 2016). The yeast *Saccharomyces cerevisiae* is currently the most widely utilized ethanol-producing microorganism on the globe (Najafpour *et al.*, 2004)

Although ethanol is synthesized by a variety of yeasts, bacteria, and fungi, *Saccharomyces cerevisiae* is the most commonly used yeast for commercial ethanol production (Tesfaw *et al.*, 2014). Due to its high ethanol output, ethanol tolerance, and ability to ferment a wide range of carbohydrates, *Saccharomyces cerevisiae* is the most commonly used microbe in ethanol production (Azhar *et al.*, 2017).

Azhar *et al.*, (2017) discussed about the effectiveness of several yeast strains in generating ethanol, with the wild-type strain producing the most ethanol. The Continuous SSF process has demonstrated its potential to produce high ethanol concentrations while maintaining a high level of productivity. The use of cell

immobilisation in the generation of ethanol was investigated. The adsorption approach is recommended for immobilising yeast cells, while calcium alginate is the ideal carrier for yeasts. Rapid cell density, simple separation from the medium, high substrate conversion, reduced inhibition, short reaction time, and cell recycling are advantages of immobilized cells in ethanol production. As a result, immobilised yeasts pave the door for a more cost-effective commercialization of bioethanol production.

Yücel *et al.*, (2015) worked over the utilization of spent tea waste (STW), a common municipal trash, as a possible substrate for producing hydrolysates for fuel ethanol synthesis. Ethanol was produced using acid pretreated STW as a substrate. Plackett–Burman designs were used to identify the essential variables that affected ethanol fermentation from STW, which were then further optimized using a five-level-three-factor central composite design using response surface approach. NH_4Cl concentration of 2.7 g/L, yeast concentration of 11.7 g/L, and temperature of 42.8°C were found to be the best conditions for ethanol fermentation. Under ideal conditions, the maximum concentrations of reducing sugar and ethanol were 28.90 g reducing sugar/L and 12.72 g EtOH/L, respectively. The quadratic polynomial equation was used to get the predicted ethanol content. In ideal conditions, the predicted ethanol content was 13.38 g EtOH/L. A verification experiment (12.72 g EtOH/L) was used to establish the validity of the projected model.

Nasir *et al.*, (2016) cultivated *Saccharomyces cerevisiae* from pineapple and orange, and investigated the effects of metal on ethanol production. Various fruit peels have been used to isolate and characterise stress-tolerant, high-potential ethanol-producing yeast strains. Two yeast isolates from pineapple (Pa) and orange (Or) were isolated, described morphologically and physiochemically, and optimized for ethanol production utilising sugarcane molasses as a substrate. Indigenous yeast isolates were discovered to be beneficial to the fuel demand and industrial alcohol industries.

Sabate *et al.*, (2002), used RFLP analysis of ribosomal genes and mitochondrial DNA to isolate and identify yeasts associated with vineyards and wineries. They looked at yeast colonies isolated from vineyard and cellar substrates. Amplification and digestion of a portion of the ribosomal RNA gene repeat unit were used to determine the species of yeast. Mitochondrial DNA restriction analysis was also used to characterize *Saccharomyces* strains. The vineyard environment was dominated by oxidative basidiomycetous yeasts with no enological potential. Grape skin yeasts vary

depending on grape varietal, vintage, and degree of grape ripening. These grape surface species formed the majority of the microbiota in must and developed throughout the early stages of the process. Some days before the harvest, yeast colonies were separated and identified from the walls of a fermentation vat. Contrary to expectations, *Saccharomyces cerevisiae* was not the most often isolated species, with *Candida sorbosa* accounting for 76% of the total. *Saccharomyces* strains were previously isolated from this cellar's wine fermentations. As a result, these strains should be thought of as permanent residents of the vineyard.

Boudjema *et al.*, (2016) studied bioethanol generation from cheese whey that has been prehydrolyzed enzymatically with galactosidase by isolated yeast strains. The yeast strains were isolated from Algerian natural sources (soil and grape) and chosen for their high ethanol tolerance and ethanol generation on prehydrolyzed cheese whey. Morphological, physiological, and biochemical parameters were used to identify the chosen ones. The D1/D2 domain of the 26S rDNA region was then amplified and sequenced for molecular identification. The operating parameters of fermentation, such as temperature, pH, and substrate concentration (glucose and galactose combination), are examined for an effective yeast strain. Three strain isolates were found to be capable of producing bioethanol among the selected and identified yeast strains. When compared to the stocked strains in the data bank, these strains are *Hanseniaspora opuntiae* Z087A0VS, *Candida tropicalis* Z087B0VS, and *Candida tropicalis* Z087D0VS, with 99 percent and 100 percent identity, respectively. Furthermore, *Hanseniaspora opuntiae* has an ethanol tolerance of up to 11%, while the two other *Candida* strains have a tolerance of up to 12%. Temperature 30°C, pH 5, and sugar concentration (glucose and galactose) of 12.5 percent (w/v) were shown to be the most effective fermentation parameters for *Candida tropicalis* Z087B0VS. They found *Candida tropicalis* Z087B0VS as a promising candidate for commercial bioethanol production.

Rahman *et al.*, (2013) isolated two ethanol producing strain of *Saccharomyces cerevisiae* from grapes and dates juice. They were examined for alcoholic fermentation using sugarcane molasses, and their growth conditions in terms of pH and sugar content were optimized. The ideal temperature for fermentation was found to be 30°C, pH 6.0, and a sugar content of 6.5%. Date juice isolation was found to be highly tolerant to temperature, pH, and a high ethanol concentration in the medium in stress tolerant tests.

S. cerevisiae isolated from date juice produced 7.75% ethanol in molasses under optimal conditions.

Current trends in bioethanol production

Chen and Fu (2016) integrated feedstock management, selective fractionation, synergistic enzymatic hydrolysis, industrial fermenting yeast strains, simultaneous saccharification and co-fermentation to explore industrial technology for bioethanol production from lignocellulosic biomass. The contribution of integrated technologies to economic feasibility was also examined through an industrial operation test. When compared to the traditional conversion process, the feedstock cost was lowered by 19.4 percent using integrated technology. Synergistic enzyme systems lowered enzyme loading by 25.0 percent when compared to single cellulase addition. Inhibitor-tolerant *Saccharomyces cerevisiae* was used to develop pre-hydrolysis, simultaneous saccharification, and co-fermentation without the need for detoxification. A concentration of more than 4% (w/w) ethanol was reached, resulting in a potential ethanol yield of 72.3 percent. Pentose conversion efficiency was increased by 41%, resulting in a 41% increase in ethanol yield. Furthermore, lignin plastic composite material (LPCM) and compressed natural gas (CNG) were co-produced, reducing the capital cost of the process and improving its economic feasibility. As a result, the overall cost of ethanol has dropped to 5571.6 Yuan per ton (Yuan, Chinese currency). Finally, the low cost of feedstock, the easy and integrated conversion process, and the ability to produce several products all contribute to the competitiveness of this integrated industrial technology for lignocellulosic bioethanol production.

Singh *et al.*, (2016) revealed that open burning of rice straw generates significant amounts of greenhouse gases such as CO_2, CH_4, and N_2O, which contribute to global warming. Rice is India's most important food crop, and rice plantation is done for food rather than fuel, therefore CH_4 emissions are already associated with it. The use of leftover rice straw for bioethanol production is a long-term waste management strategy. Straw transportation, chemical and enzyme use, by-product combustion, and ethanol fuel combustion are major sources of GHG emissions, while GHG savings come from power generation, rice straw avoidance, and fossil fuel avoidance. Therefore, although, there is an emission of GHG gases during the conversion of rice straw into bioethanol but, it is very low as compared to the fossil fuels and due to this, it is better than fossil fuels. Bioethanol is a clean, renewable, and sustainable fuel, but commercial production poses a significant obstacle. At the farm, there is a need to

improve nitrogen use. More research should be done on increasing ethanol output so that it can eventually replace coal and gasoline as the energy feedstock in ethanol plants. Using rice straw for bioethanol not only solves the problem of how to dispose of waste, but it also improves the socioeconomic standing of rural people.

Conde-Mejia *et al.*, (2012) provide the results of a comparison of numerous pretreatment strategies for producing bioethanol from lignocellulosic materials. First, a screening of the numerous options documented in the literature resulted in the selection of six options, which were then assessed from an energy consumption standpoint. Other elements were then added to the screening process. Inhibitions, as well as chemical and water usage, are among these issues. The actual results were compared to the theoretical production goals. The unit cost for the possible biorefinery owing to the pretreatment stage was calculated by combining the cost of each pretreatment method with the computation of production targets based on stated yields. The steam explosion and dilute sulfuric acid hydrolysis procedures were identified as having the lowest energy requirements in the initial analysis with no sort of integration. Steam explosion, liquid hot water, dilute sulfuric acid hydrolysis, and ammonia fibre explosion became more energy-efficient technologies after the recycling was introduced. SE, on the other hand, was determined to be a less favourable pretreatment technique from the standpoint of inhibition and waste minimization. The costs of DA and AFEX have been found to be quite sensitive to chemical prices.

Tomas-Pejo *et al.*, (2008) discuss the current state of enzyme-based bioethanol synthesis, focusing on several process centrifugation and drawbacks that must be overcome for a successful industrial process. In this regard, commercial-scale stream explosion (SE) pretreatment proved the viability of pretreated lignocellulosic biomass (LB) in an industrial ethanol production process. For a successful industrial process for ethanol generation from lignocellulose, high substrate loading, sugar recovery after pretreatment, tolerance to inhibitory chemicals, and Xylose fermentation by yeast must all be adjusted. Furthermore, for an economically viable approach, lower enzyme and yeast loading is essential. To combat the presence of harmful substances in fermentation broth, genetic alteration, evolutionary engineering, and adaptive strategies are all viable options for producing more tolerant yeast. Because the filtering stage after pretreatment is omitted and the amount of waste water as well as freshwater requirements are decreased when employing complete pretreated materials, operational costs can be minimized.

Chapter-3

Materials and Methods

CHAPTER 3

MATERIAL AND METHOD

The present research work was carried out in the PG laboratory of Division of Microbiology, Faculty of Basic Sciences located at Sher-e-Kashmir University of Agricultural Sciences and Technology of Jammu. Materials and Methods used for the experiments are discussed as under:

3.1 Collection of samples from fruit and dairy sources

Fruit sample required for the experiments were collected from the local markets of Jammu District. Fruit sampled for the isolation of *Saccharomyces cerevisiae* were Grapes, Apples, Orange, Pineapple, Papaya and Sugarcane. Kaladi, a fermented milk product was also collected from the local markets of District Udhampur for the studies.

The collected fruit and Kaladi sample were brought in the laboratory of Division of Microbiology, Faculty of Basic Sciences at Sher-e-Kashmir university of Agricultural Sciences and Technology of Jammu and stored at 4ºC in refrigerator for further studies.

Lyophilized *Saccharomyces cerevisiae* MTCC-170 was procured from IMTECH- Chandigarh that was used as a check. This culture was revived on YEPD slants and incubated at 30ºC for 24h. It was stored at 4ºC for further use.

3.2 Sterilization of glassware

Glassware was washed with distilled water and allowed to dry for all the laboratory experiments. The sterilization of glassware was done before use in hot air oven at 180ºC for 25 minutes.

3.3 Preparation of media

For the isolation and screening of isolates three media were used namely YMA (Yeast Mannitol agar), YEPD (Yeast extract -10g/L, peptone-20g/L, dextrose-20g/L and agar-15 g/L) and Screening media (peptone- 10g, dextrose-5g, phenol red, and NaCl-5g in 1 Lt of distilled water).

Sterilization of 500 mL of conical flask was done in hot air oven at 180ºC for 25 minutes. 250 mL of prepared media components was added in a 500 mL of sterile conical flask and autoclaved at 121ºC,15 psi for 15 minutes before the experiment.

3.4 Isolation and Purification of *Saccharomyces cerevisiae*

3.4.1 From fruit samples

The collected fruit samples were washed under tap water. After cutting it into small pieces it was kept in sterile Petri plate at 30ºC for 96 h. 1g of peal was subjected to serial dilution method (Plate 1).

3.4.1.1 Serial dilution method

In serial dilution, sample was diluted through a set of standard volumes of sterile diluents, which include distilled water or saline 0.9% concentration. 8 clean and sterile culture tubes were taken. 1 culture tube was filled with 10 mL of distilled water while the remaining with 9 mL of distilled water and further autoclaved at 121ºC, 15psi for 15 minutes. Stock solution was prepared by adding 1g of crushed peal of fruit sample into 10 mL of autoclaved distilled water in a culture tube. This stock solution was used for making dilutions of 10^{-1} to 10^{-7} in culture tubes. 1mL of stock solution was transferred into 1st culture tube having 9 mL of distilled water with the help of sterilized micropipette, it makes a dilution of 10^{-1}. Sample was mixed properly by shaking the culture tube. Again, 1mL of mixture sample was transferred from 10^{-1} to 2nd culture tube having 9 mL of distilled water to form 10^{-2} dilution. Repeat the steps for the remaining tubes to form dilutions upto $10^{-7}.10^{-5}$ and 10^{-7} dilution was used for spreading over YMA (Yeast Mannitol agar) plate and incubated it at 30°C for 24 h in a BOD incubator.

Selected colonies were transferred to fresh YMA plate. Pure cultures were obtained through streak plate method (Plate 2). Isolated pure culture was transferred to fresh YMA slants and stored at 4°C for further studies.

3.4.2 From dairy samples

Milk product (Kaladi) was cut in small pieces. It was followed by Serial dilution technique. 8 clean and sterile culture tubes were taken. 1 culture tube was filled with 10 mL of distilled water while the remaining with 9 mL of distilled water and further autoclaved at 121ºC, 15psi for 15 minutes. Stock solution was prepared by

adding 1g of mashed kaladi sample into 10 mL of autoclaved distilled water in a culture tube. This stock solution was used for making dilutions of 10^{-1} to 10^{-7} in culture tubes. 1mL of stock solution was transferred into 1st culture tube having 9 mL of distilled water with the help of sterilized micropipette, to make a dilution of 10^{-1}. Samples were mixed properly by shaking the culture tube. Again, 1mL of mixture sample was transferred from 10^{-1} to 2nd culture tube having 9 mL of distilled water to form 10^{-2} dilution. Repeat the steps for the remaining tubes to form dilutions upto 10^{-7}.10^{-5} and 10^{-7} dilution was used for spreading over YMA (Yeast Mannitol agar) plate and incubating it at 30°C for 24 h in a BOD incubator.

Selected colonies were transferred to fresh YMA plated. Pure cultures were obtained through streak plate method. Isolated pure culture was transferred to fresh YMA slants and stored at 4°C for further studies.

3.5 Characterization of Isolated cultures

For the characterization of isolated cultures, morphological and microscopic characteristics were examined.

3.5.1 Morphological characterization

Isolated cultures were allowed to grow on YEPD (Yeast extract -10g/L, peptone-20g/L, dextrose-20g/L and agar-15g/L) agar plate for studying the morphological characteristics. Individual isolated culture was streaked over YEPD agar plates with the help of sterilized inoculation loop. The plates were incubated at 30°C for 24 h and analysed visually for colony characteristics which include color, texture, margins etc.

3.5.2 Microscopic characterization

Freshly grown cultures on YEPD Medium were examined under microscope in order to study the microscopic characteristics. They were analysed for the shape and size of cells. Isolated yeast cells were stained using Lactophenol Cotton Blue Staining Method. A clean and grease free slide was taken, and a drop of lactophenol cotton blue statin was placed over the slide. With the help of flamed and cooled inoculating loop, a small drop of 24 h old isolated cultures on YEPD agar plate were placed into Lactophenol cotton blue drop. It was finally spread to form a thin layer using another sterile inoculation loop. Finally, a coverslip was placed over the thin layer. First it was

observed under 10x and then at 40x magnification under compound microscope in order to record the microscopic characteristics.

3.6 Screening for Bioethanol production

After the identification of isolates, screening was performed. The screening of isolates was based on the ethanol producing ability of *Saccharomyces cerevisiae* on screening media. Screening medium was prepared by adding 10 g of peptone, 5 g dextrose, phenol red, and 5g NaCl in 1 Lt of distilled water. The screening media is red due to the presence of phenol red indicator and a change in color from red to yellow after fermentation indicates the production of ethanol from the Isolates. 15 mL of screening media was poured into 30 mL of screw capped tubes which was autoclaved at 121ºC and 15 psi for 20 minutes. Isolates were transferred to fresh YEPD slants with the help of sterilized inoculating loop and incubated at 30ºC for 48 h in a BOD incubator. 48 h old isolated culture was used to inoculate autoclaved fermentation medium and kept for fermentation under static conditions at 30ºC for 72 h (Nasir *et al.*, 2017).

3.7 Quantification of Isolates for Ethanol production

In order to select high bioethanol producing isolate from the selected isolates quantification was done spectrophotometrically.

Screened isolates were transferred to fresh autoclaved YEPD slant with the help of sterile inoculating loop. After 24 h of growth, it was used for the preparation of inoculum. YEPD broth (Yeast extract -0.5%, peptone 1% and Dextrose 2%) was used as inoculation medium. In a 250 mL flask, 50 mL of inoculation medium was prepared and autoclaved at 121°C and 15 psi. For the preparation of inoculum, loop full of 24 h old culture was used to inoculate the inoculum media, which was incubated at 30°C for 24 hours at 120 rpm in an incubator shaker. YEPD broth (1% yeast extract, 1% peptone, and 12% Dextrose) was used as fermentation media. 20 mL of fermentation medium was poured into 30 mL screw-capped tubes and autoclaved at 121ºC at 15 psi for 20 min. Inoculation of screw capped tubes was done in sterilized conditions @ 10 % with the inoculum prepared and incubated at 30ºC under stationary conditions for 72h. Ethanol fermentation was also executed by MTCC 170 culture in YEPD medium as a control (Plate 3).

3.7.1 Spectrophotometry

Ethanol content produced in the fermentation medium was determined after various time intervals from 24 to 72 h using spectrophotometer. Acidified potassium dichromate was prepared by adding 34 g of potassium dichromate in 500 mL of concentrated sulfuric acid and raising the volume to 1 L with distilled water. This was stored at 4ºC for further use.

Ethanol was used as a standard. Stock solution having 1% (v/v) Ethanol was prepared. Standard ethanol solutions from 0.01% to 0.1% was prepared using serial dilution with water from the stock solution in test tubes.

A 3.0 mL of acidified potassium dichromate was incubated with 500 µL of standard ethanol solutions at room temperature for 30 min and absorbance was analyzed at 590nm wavelength using spectrophotometer. A standard curve was prepared by plotting absorbance obtained against the respective ethanol concentrations and an equation was obtained.

After 24h, 48h and 72h of fermentation ethanol content was determined by adding 10 µL of fermentation medium in a test tube and raising the volume upto 500 µl by adding distilled water. In the test tube, 3 mL of initially prepared acidified potassium dichromate was added and incubated at room temperature 30ºC for 30 minutes. After that 3 mL of the solution prepared was filled in the cuvette and record the absorbance of the solution at 590nm wavelength. Distilled water was taken as a blank solution for measuring the optical density. The ethanol concentration of the fermentation media was calculated by substituting the absorbance value obtained into the equation of the standard curve.

3.8 Pre- treatment of Agro-waste

3.8.1 Raw Material

The rice straw was collected from Chatha Research Farm and dried at 60ºC. The dried rice straw was cut into small pieces and blended in a blender to obtain uniform size. It was further pretreated in order to expose the sugar content so that it can be utilized by *Saccharomyces cerevisiae* for the production of ethanol (Plate 4). Rice straw is composed of 38% cellulose, 25% hemicellulose and 12% lignin (Hung *et al.*, 2020). Pretreatment helps to break down the rigidity of lignocellulosic biomass so

that the lignin, hemicellulose, and cellulose molecules may be exposed, allowing the yeast to access fermentable sugars (Toor *et al.*, 2020).

3.8.2 Delignification of the rice straw by alkaline pre-treatment

Blended rice straw was subjected to alkaline treatment for the removal of lignin. It would help to expose the cellulosic content. Alkaline pre-treatment involves use of KOH solution. Weight 5 g of blended rice straw in a 500 mL beaker and then 50 mL of 0.2 M KOH solution was added and finally incubated at 35ºC for 4 h. After that, it was filtered with muslin cloth and the biomass was rinsed several times under tap water to achieve a neutral pH (Yadav *et al*., 2011).

3.8.3 Acid Pretreatment of the De-lignified rice straw

The acid pre-treatment of the delignified rice straw was done in a biphasic manner using sulfuric acid. Acid hydrolysis was performed to the delignified rice straw in two levels. The first phase acid hydrolysis was done by taking the delignified rice straw in 1000 mL beaker and then 100 mL of 1% Sulphuric acid was added and finally it was incubated at 121°C for 15 minutes. It was filtered and the solid residual were used for second phase acid hydrolysis. In Second phase hydrolysis the solid residues obtained after first phase of acid hydrolysis was taken in a 1000 mL of beaker. 100 mL of 2% Sulphuric acid was added into the beaker and it was finally incubated at 121°C for 15 minutes. Solid and liquid portions were further separated through filtration using whatman filter paper. Acid pretreated biomass was dried at 40ºC and further subjected to enzymatic pretreatment (Yadav *et al*., 2011).

3.8.4 Enzymatic pre-treatment

Biphasic acid pretreatment (1% SA, 2% SA) along with enzymatic saccharification was more efficient for saccharification of rice straw (Gupta *et al.*, 2015). Enzymatic saccharification was done by using cellulase enzyme. The dried acid pretreated biomass was weighed and pre inoculated with acetate buffer having concentration of 50 mM and pH, 5.6 in a 150 mL of sterile flask for 15 min at 37°C. Acetate buffer was added at 10% substrate consistency. After that cellulase loading of 15 CMcaseIU/G was added and further incubated at 50 °C and 150 rpm for 48 h in a water bath. Finally, the liquid and solid fractions were separated using whatman filter paper.

Prehydrolysate which include the liquid fraction obtained after alkaline and acidic pretreatment and hydrolysate obtained after enzymatic pretreatment were mixed for ethanol fermentation as total hydrolysate and analysed for reducing sugar content by DNSA (3, 5- Dinitrosalicylic Acid) method (Miller Gl, 1985).

3.9 DNSA Test

Table 1: DNSA reagent Components

S.No.	Chemicals	Percentage(w/v)
1	3,5-Dinitrosalicylic acid	1
2	Phenol	0.2
3	Na_2SO_3	0.05
4	NaOH	5

DNSA reagent was prepared by mixing 3,5-Dinitrosalicylic acid (1%w/v), Phenol (0.2%w/v), Na_2SO_3 (0.05%w/v) and NaOH (5%w/v) and raised the volume up to 100 mL by adding distilled water. DNSA reagents was finally stored in dark colored bottles for further use (Table 1).

For the estimation of reducing sugars, 100µl of the sugar hydrolysate were mixed with 900µl of distilled water in test tube and finally incubated with 1 mL of DNSA reagent at 95°C for 10 min in a water bath. 1mL of NaK Tartrate (40% w/v) was added with the help of micropipette after that content was cooled under tap water and absorbance was analysed at 575nm (Plate 6).

A standard curve for glucose was formed and the concentration of glucose was determined by comparing OD with this curve. Initial sugars and final residual sugars were estimated.

3.10 Ethanol fermentation of the Rice Straw Hydrolysate

Ethanol Fermentation of the total Rice Straw Hydrolysate obtained after pretreatments were performed using best isolate of yeast culture (*Saccharomyces cerevisiae)*, MTCC-170 culture.

For the production of bioethanol from rice straw hydrolysate Ethanol production medium (EPM) having pH 5.6 was prepared. EPM medium was prepared by adding peptone-1 g/L, yeast extract-1.5g/L, dipotassium phosphate-1g/L, ammonium sulphate-1 g/L and magnesium sulphate-1g/L into rice straw hydrolysate in a 500 mL flask. 20 mL of the EPM medium prepared was finally added into 30 mL of screw capped tubes and autoclaved at 121ºC, 15psi for 15 minutes (Vaid *et al.*, 2017). The media was inoculated with 24 h old yeast cultures at the rate of 1.5% (v/v), and incubated at 30ºC. Ethanol content was determined after various time intervals from 24 to 72 h spectrophotometrically as discussed earlier.

Experimental ethanol yield and Fermentation efficiency was calculated using the formula in equation no. 1 and 2 (Vogel *et al.*, 2011).

All the experiments were conducted in triplicates, and data presents the mean values.

$$\text{Ethanol from hexoses and pentoses(mL/g)} = \frac{1.1\times 0.51 \text{ ethanol per glucose}}{\text{Specific volume of ethanol (0.789 g/mL)}} \quad (1)$$

$$\text{Fermentation efficiency (\%)} = \frac{\text{Experimental yield} \times 100}{\text{Theoretical yield}} \quad (2)$$

3.11 Optimization of culture conditions for bioethanol production

S. cerevisiae is capable of very rapid rates of glycolysis and ethanol production occur under optimal condition The culture conditions of high ethanol producing isolate was optimized which include pH, Temperature and Agitation. Classical one factor at a time was used for the optimization parameters which involves change in only one factor at a time and all the other factors are kept constant. To examine the effects of temperature, initial pH and agitation on ethanol production, isolates were cultivated at a range of temperatures 25, 30, 35ºC; various pH 4.6, 5.6 and 6.6 and various agitation rate 0, 120, 150 rpm.

3.11.1 pH optimization

In a 250 mL flask, 50 mL of inoculation medium (Yeast extract -0.5%, peptone 1% and Dextrose 2%) was prepared and autoclaved at 121°C and 15 psi. For the

preparation of inoculum, loop full of 24 h old culture was used to inoculate the inoculum media, which was incubated at 30°C for 24 hours at 120 rpm in an incubator shaker. YEPD broth (1% yeast extract, 1% peptone, and 12% Dextrose) was used as fermentation media having initial pH of 4.6, 5.6 and 6.6. 20 mL of fermentation medium was poured into 30 mL screw-capped tubes and autoclaved at 121ºC at 15psi for 20 min. Inoculation of screw capped tubes was done in sterilized conditions @ 10 % with the inoculum prepared and incubated at 30ºC under stationary conditions for 72 h. Concentration of ethanol was measured spectrophotometrically at 24, 48 and 72 h.

3.11.2 Temperature optimization

In a 250 mL flask, 50 mL of inoculation medium (Yeast extract -0.5%, peptone 1% and Dextrose 2%) was prepared and autoclaved at 121°C and 15 psi. Inoculum is prepared by adding loop full of 24 h old culture to the inoculum media, which was incubated at 30°C for 24 hours at 120 rpm in an incubator shaker. 20 mL of fermentation medium broth (1% yeast extract, 1% peptone, and 12% Dextrose) having initial pH of 5.6 was poured into 30 mL screw-capped tubes and autoclaved at 121ºC at 15psi for 20 min. Inoculation of screw capped tubes was done in sterilized conditions @ 10 % with the inoculum prepared and incubated at 25ºC, 30ºC and 35ºC under stationary conditions for 72 h. Concentration of ethanol was measured spectrophotometrically at 24, 48 and 72 h.

3.11.3 Agitation

In a 250 mL flask, 50 mL of inoculation medium (Yeast extract -0.5%, peptone 1% and Dextrose 2%) was prepared and autoclaved at 121°C and 15 psi. for the preparation of inoculum, loop full of 24 h old culture was used to inoculate the inoculum media, which was incubated at 30°C for 24 hours under shaking conditions. 100 mL of fermentation medium broth (1% yeast extract, 1% peptone, and 12% Dextrose) having initial pH of 5.6 was poured into 500 mL of flask and autoclaved at 121ºC at 15psi for 20 min. Inoculation of flask was done in sterilized conditions @ 10 % with the inoculum prepared and incubated at 30ºC under static conditions, 120 rpm and 180 rpm for 72 h. Concentration of ethanol was measured spectrophotometrically at 24, 48 and 72 h.

3.12 Quality parameters

Quality parameters include Initial glucose concentration in the total hydrolysate before fermentation, final glucose concentration after 72 h of fermentation, Percentage of Sugar consumption, Fermentation efficiency, Ethanol yield per gram of sugar consumed and Volumetric productivity.

Initial and final glucose concentration of the EPM was determined by DNSA method as discussed earlier. Percentage of sugar consumption was calculated by taking the difference of final and initial sugar concentration then diving it with initial sugar content and finally multiplied by 100 (Rezania *et al.*, 2018). Ethanol yield per gram of sugar consumed is the amount of ethanol produced(mg/mL) divided by the sugar consumed. Ethanol volumetric productivity was calculated by dividing the ethanol concentration(g/L) of the fermentation medium with the fermentation time in hours. It was expressed as volumetric productivity (g/L/h) (Rezania *et al.*, 2018).

A

B

C

D

E

F

Plate 1: Fruit samples for the isolation of *Saccharomyces cerevisiae*

A: Sugarcane, B: Apple, C: Orange, D: Papaya, E: Pine apple and F: Grapes

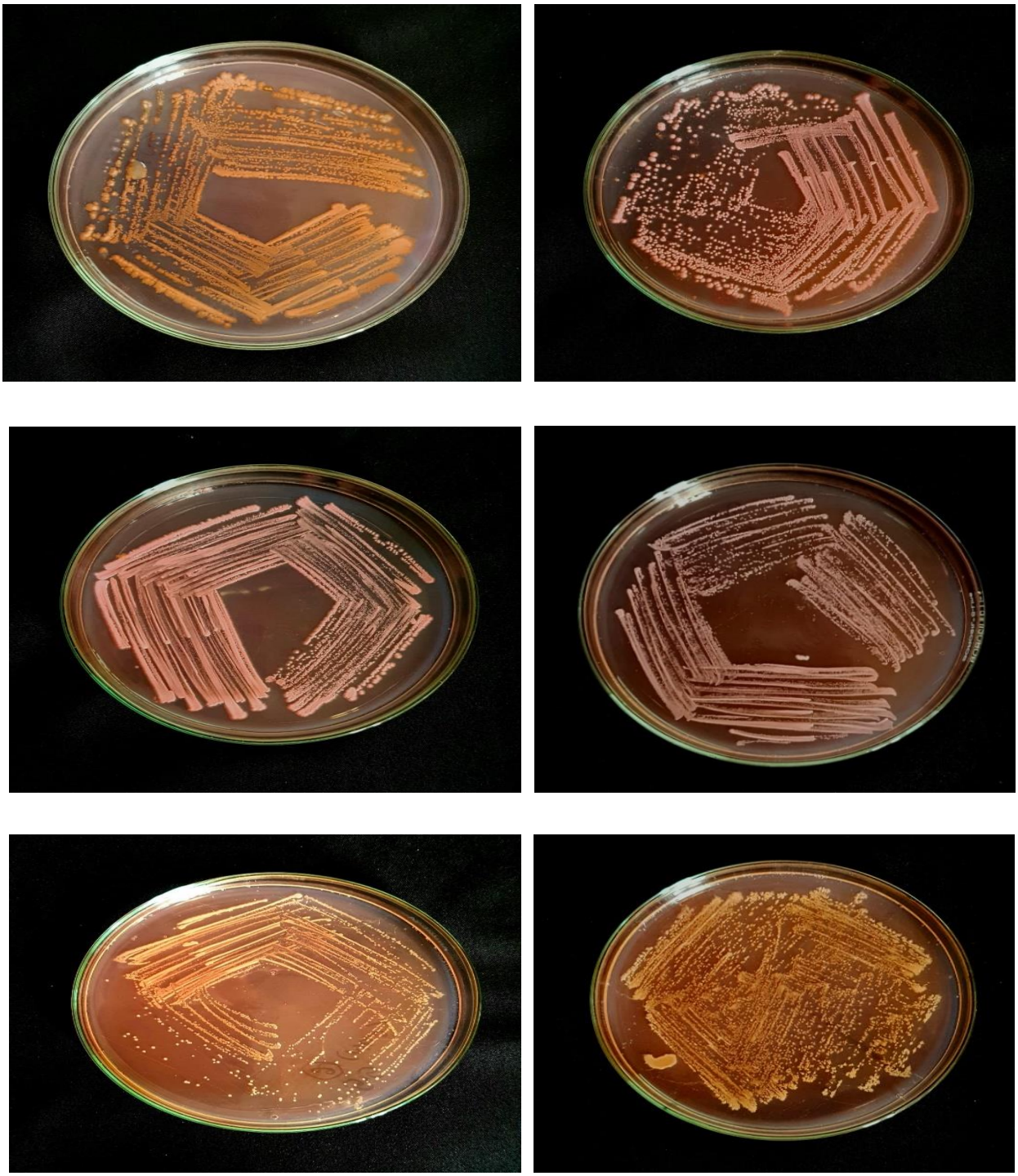
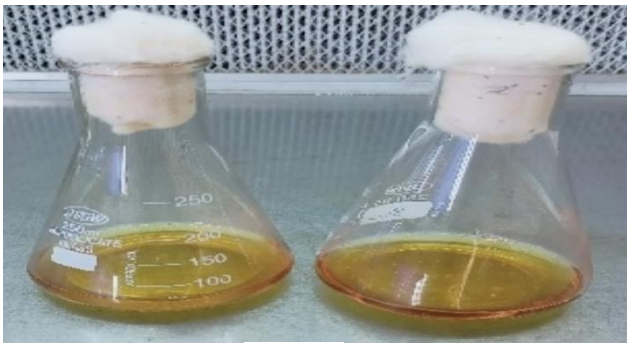
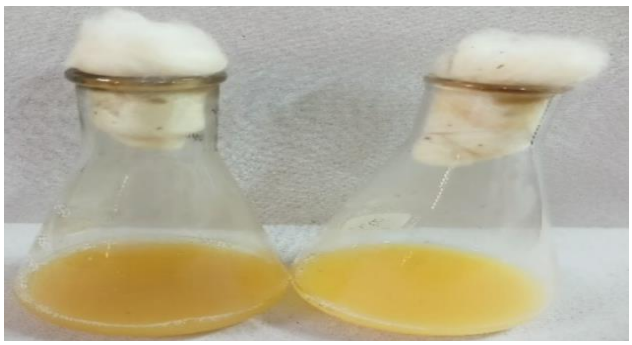


Plate 2: Pure cultures obtained by streaking on YMA agar media



A



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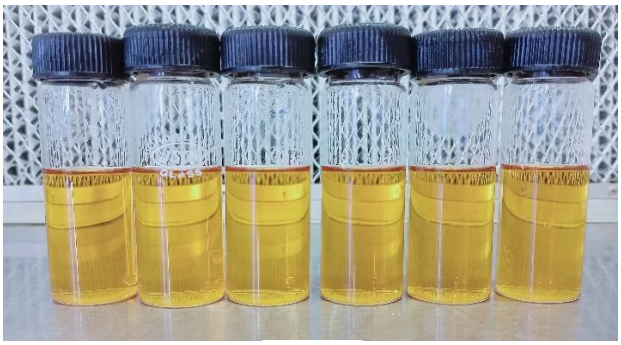
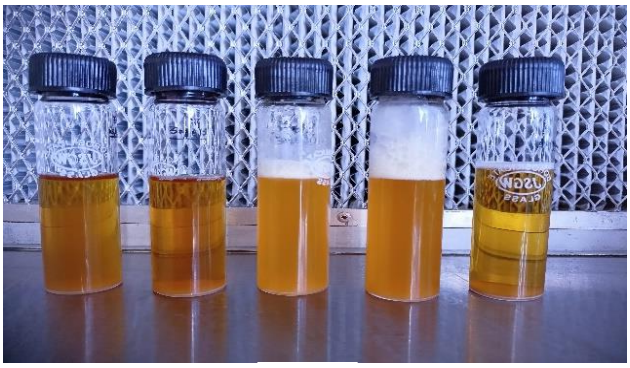


D



E

F

Plate 3: Quantification of ethanol produced from the isolates on YEPD media

A: Inoculation media

B: Inoculation media after incubation of 24 h

C: Inoculation of fermentation medium

D: Fermentation media

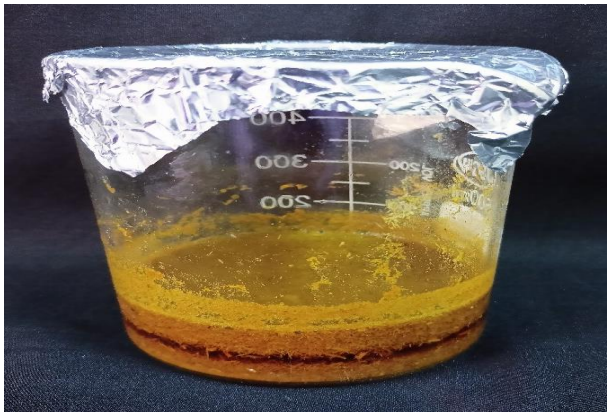
E: Fermentation media after 72 h

F: Spectrophotometric analysis of fermentation broth

A

B

C



D

E

Plate 4: Pretreatment of Rice straw

A: Rice straw collected from Chatha research farm

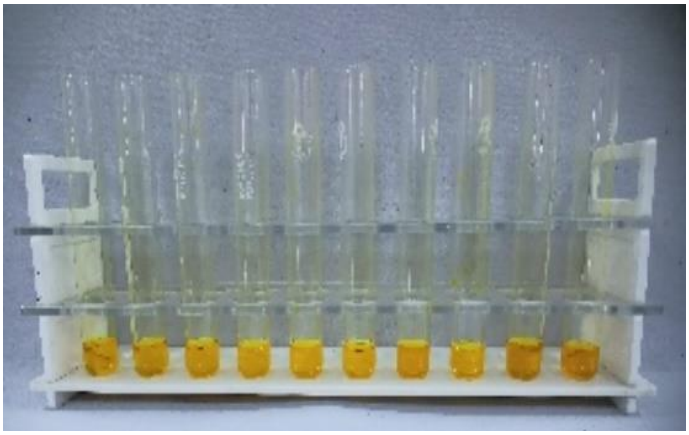
B: Rice straw cut into small pieces

C: Blended rice straw

D: Alkaline pretreatment of Rice straw

E: Acidic pretreatment of Rice straw

A



B

Plate 5: DNSA test of total rice straw hydrolysate for the estimation of reducing sugars

A: Total hydrolysate obtained by pretreatment of Rice straw

B: Spectrophotometric analysis of DNSA Test

Chapter-4

Results

CHAPTER 4

RESULTS

In the present study *Saccharomyces cerevisiae* was isolated from fruit and kaladi samples. The potential isolates were identified on the basis of morphological and microscopic characters. They were further screened on the basis of ethanol producing potential of *Saccharomyces cerevisiae*. Quantification on YEPD media was done spectrophotometrically. Selected isolate was used for the fermentation of rice straw after several pretreatments and quantification was done. Optimization of culture condition of best isolate was also done.

4.1 Isolation and purification of *Saccharomyces cerevisiae*:

Isolation of *Saccharomyces cerevisiae* was done from fruit and dairy samples collected from local market of Jammu and Udhampur district respectively. Fruit samples include Apple, Orange, Pineapple, Papaya, Grapes and Sugarcane while Kaladi was taken as dairy sample. Total fifteen isolates (three from sugarcane, two from pineapple, two from grapes, one from orange, one from papaya, one from apple and five from kaladi) were obtained from the collected samples (Table 2).

4.2 Microscopic and colony characteristic

In the present study microscopic and colony characteristics were examined based on the growth of isolates on YEPD media. Isolated cultures show smooth texture, entire margins and whitish or off-white color (Plate 6). Microscopic characteristics show ovoid and elongated cells (Plate 7, Plate 8). The morphological and colony characteristics were compared with that of MTCC 170 strain of *Saccharomyces cerevisiae* (Table 3).

4.3 Screening of potential isolates for bioethanol production

The potential isolates were screened for the production of bioethanol. They were allowed to grow in the screening medium for about 72h and analysed for the color change. The change in the color of screening medium after 72h of fermentation from red to yellow indicate the production of bioethanol. The results presented in table 3 show that 15 out of 14 (S-1, S-2, S-3, S-4, S-5, S-7, S-8, S-9, S-9, S-10, K-1, K-2, K-3, K-3, K-4 and K-5) show a positive color change and indicate the production of ethanol (Table 4, Plate 9)

4.4 Quantification of Isolates for bioethanol production

Quantification of screened isolates was done spectrophotometrically. Ethanol was used as a standard. Isolates were grown on YEPD medium and ethanol content was estimated after various time intervals from 24 to 72 h of fermentation using acidified potassium. Table 5 shows ethanol concentration of isolates at 24h, 48h and 72h of fermentation. S-9 isolate show maximum ethanol concentration of 17.88 mg/mL and fermentation efficiency of 69.78% on YEPD media (Table 5, Fig. 1).

4.5 Estimation of initial reducing sugar content by DNSA method.

Total sugar hydrolysate obtained after various pretreatments were analyzed for the content of reducing sugar by DNSA method. It helped us to know the efficiency of pretreatment and the quantity of fermentable sugars exposed for yeast to act. Initial glucose concentration of total hydrolysate was 7.05±0.4922 mg/mL.

4.6 Estimation of ethanol content after fermentation with the rice straw hydrolysate

Ethanol content was determined spectrophotometrically after the fermentation with S-9 culture using Rice straw hydrolysate. MTCC 170 culture was used as a control. Results in table 6 show that S-9 culture maximum ethanol concentration of 12.26 mg/mL and fermentation efficiency of 69.5% at 24h. The concentration of ethanol produced by S-9 isolate reduces to 7.37 mg/mL and 2.01 mg/mL after 48h and 72h of fermentation, respectively. MTCC-170 culture produced maximum ethanol concentration of 14.98 mg/mL and fermentation efficiency of 84.9% at 24h the concentration of ethanol produced by MTCC 170 also decreases to 6.38mg/mL and 1.29 mg/mL after 48h and 72h of fermentation respectively (Table 6).

4.7 Estimation of Final reducing sugar content by DNSA method

The final reducing sugar content of the fermentation media was analysed by DNSA method. S-9 and MTCC 170 culture show the final glucose concentration of 3.41±0.046 mg/mL and 2.32±0.032 mg/mL respectively after 72h of fermentation.

4.8 Optimization of culture conditions

Optimization of culture conditions for the growth of S-9 isolate includes temperature, pH, and agitation using classical factor one at a time. Three factors were analysed for each character. Ethanol concentration was determined

Table 2: Isolates obtained from Fruit and Kaladi sample

S.No.	SAMPLES	ISOLATES
1	**SUGARCANE**	S-1, S-5, S-7
2	**PINEAPPLE**	S-2, S-9
3	**GRAPES**	S-3, S-10
4	**ORANGE**	S-6
5	**PAPAYA**	S-8
6	**APPLE**	S-4
7	**KALADI**	K-1, K-2, K-3, K-4, K-5

Table 3: Colony and Microscopic characteristics of Isolates from fruit and kaladi sample

S.No.	Isolates	Colony characteristics	Microscopic characteristics
1	S-1	Smooth, Whitish, Entire Margin	Ovoid, Elongate Cells
2	S-2	Smooth, Entire Margin, Off-White	Ovoid Cells
3	S-3	Off-White, Entire Margin, Whitish	Ovoid, Elongated Cells
4	S-4	Whitish, Smooth, Entire Margin	Ovoid, Elongated Cells
5	S-5	Smooth, Entire Margin, Off-Whitish	Elongated, Ovoidcells
6	S-6	Smooth, Entire Margin, Whitish	Ovoid Cells
7	S-7	Smooth, Entire Margin, Creamish	Ovoid Cells
8	S-8	Off-White, Smooth, Entire Margin	Ovoid Cells
9	S-9	Smooth, Whitish, Entire Margin	Ovoid Cells
10	S-10	Cremish, Smooth, Entire Margin	Elongated, Ovoid Cells
11	K-1	Smooth, Whitish, Entire Margin	Ovoid Cells
12	K-2	Smooth, Entire Margin, Cremish	Ovoid Cells
13	K-3	Off-White, Smooth, Entire Margin	Ovoid Cells
14	K-4	Smooth, Cremish, Entire Margin	Ovoid Cells
15	K-5	Smooth, Entire Margin, Cremish	Ovoid Cells

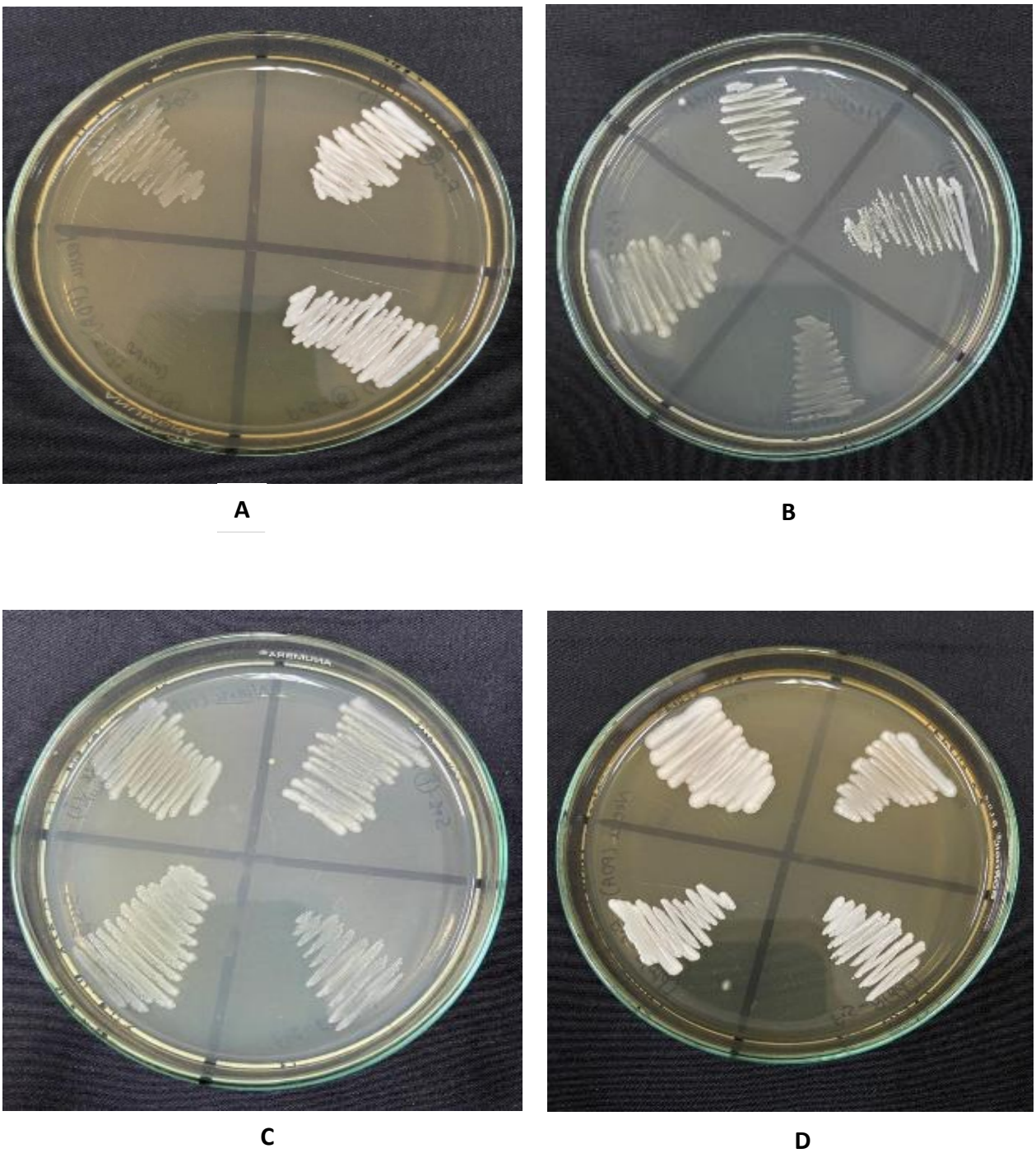


Plate 6: Colony characterization of isolates of *Saccharomyces cerevisiae* obtained from fruit and kaladi samples on YEPD media

A: K-4, S-6 and S-9 Isolates

B: K-5, S-10, S-7 and K-2 Isolates

C: S-3, S-5, K-3 and S-2 Isolates

D: S-8, S-1, S-4 and K-4 Isolates

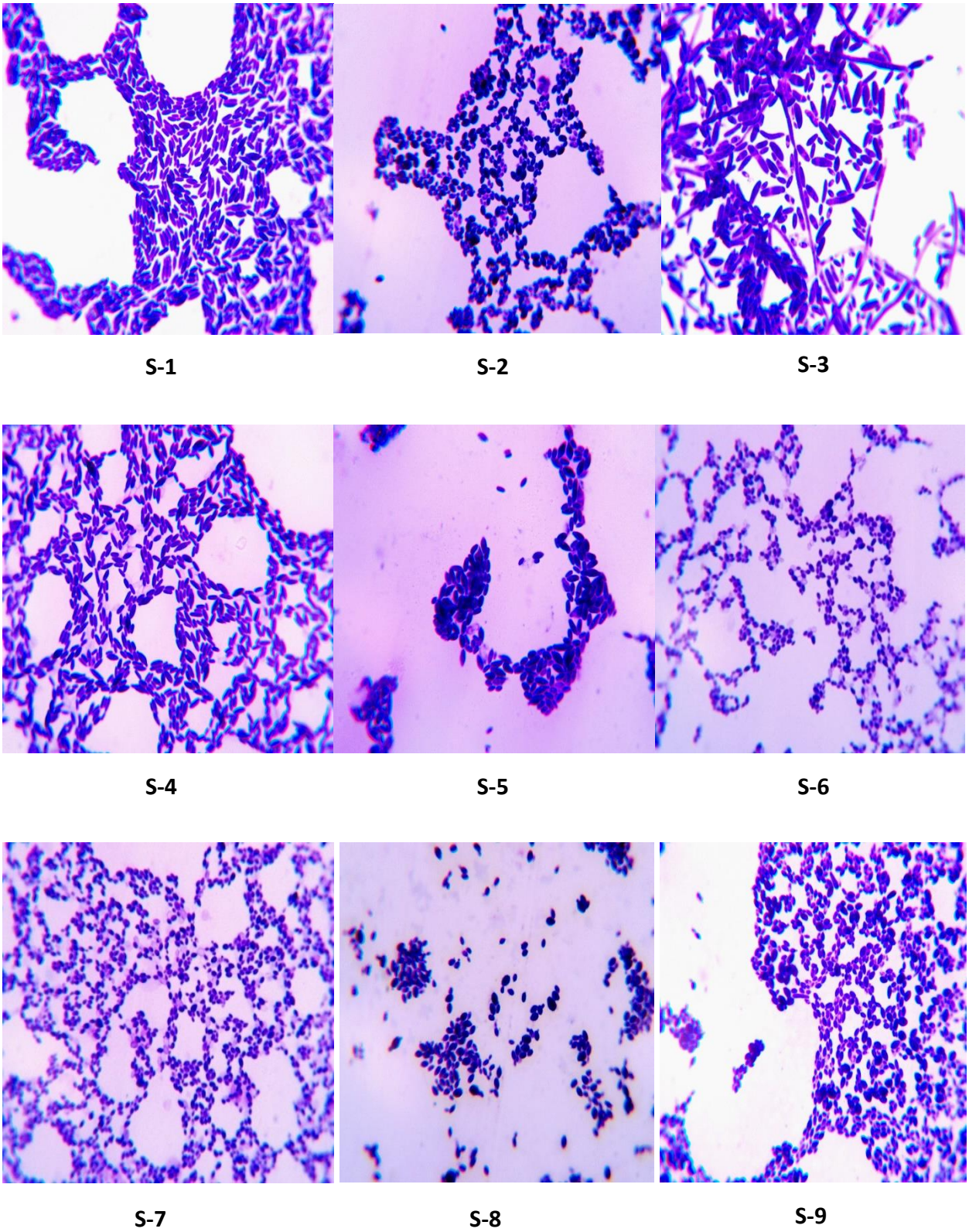
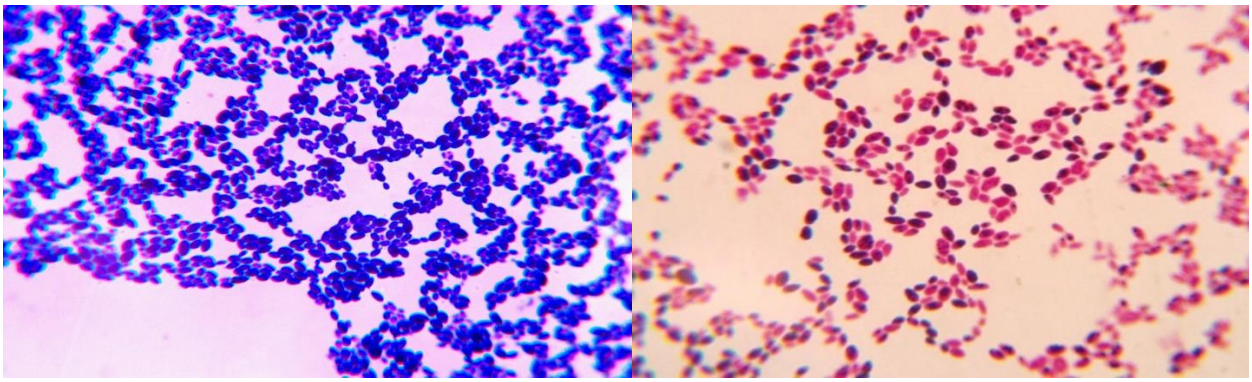
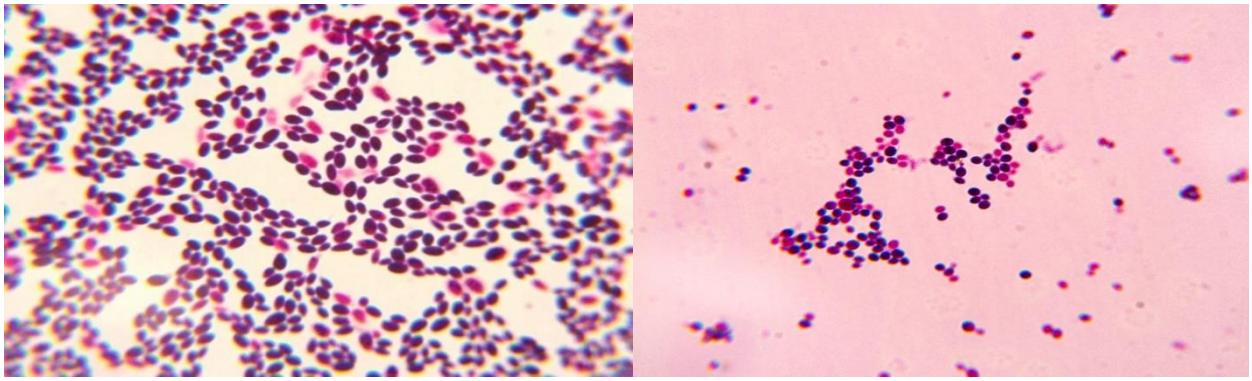


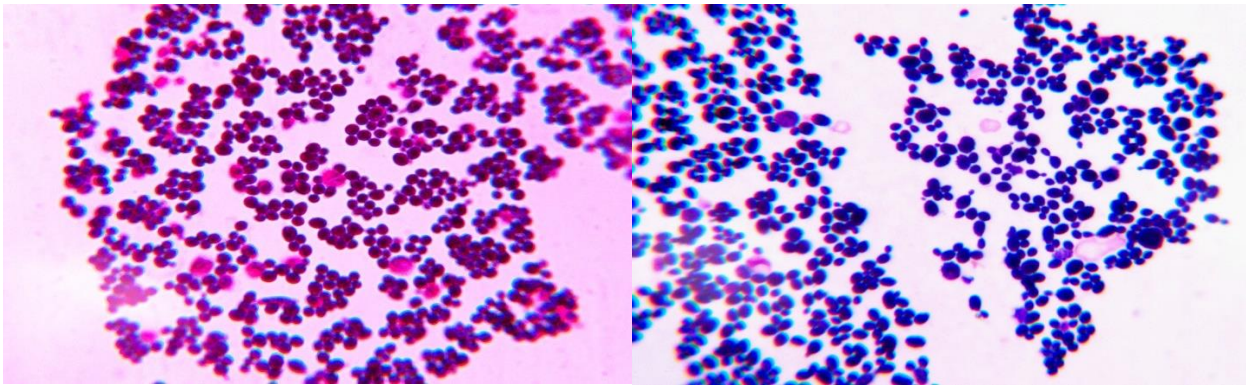
Plate 7: Microscopic Characteristics of S-1, S-2, S-3, S-4, S-5, S-6, S-7, S-8 and S-9 isolates obtained from fruit samples on YEPD media



S-10 K-1



K-2 K-3



K-5 K-5

Plate 8: Microscopic Characteristics of S-10, K-1, K-2, K-3, K-4 and K-5 isolates obtained from fruit sample and kaladi sample on YEPD media

Table 4: Screening of potential isolates and MTCC 170 culture for bioethanol production on screening media

S.No.	Isolates	Color change in Screening media after fermentation of 72 h
1	S-1	+
2	S-2	+
3	S-3	+
4	S-4	+
5	S-5	+
6	S-6	-
7	S-7	+
8	S-8	+
9	S-9	+
10	S-10	+
11	K-1	+
12	K-2	+
13	K-3	+
14	K-4	+
15	K-5	+
16	MTCC-170	+

A

B

C

Plate 9: Screening of potential isolates and MTCC 170 culture for bioethanol production on screening media

A: Screening media before fermentation

B and C: Screening media after fermentation of 72 h with the isolates

Table 5: Estimation of ethanol concentration of isolates and MTCC 170 culture spectrophotometrically

S.No.	Culture	Ethanol concentration (mg/mL)			Maximum ethanol concentration (mg/mL)	Fermentation efficiency %
		24hr	48hr	72hr		
1	S-1	4.77±0.225	11.38±0.290	8.99±0.322	14.77±0.225	57.8
2	S-2	12.38±0.232	10.04±0.389	4.68±0.340	12.38±0.232	48.4
3	S-3	11.62±0.225	7.3±0.612	3.6±0.389	11.62±0.225	45.47
4	S-4	15.32±0.229	11.56±0.290	5.95±0.484	15.32±0.229	59.98
5	S-5	1.18±0.318	8.15±0.389	5.15±0.225	8.15±0.389	31.89
7	S-7	10.14±0.389	7.59±0.385	3.51±0.393	10.14±0.389	39.68
8	S-8	15.72±0.304	12.32±0.410	5.64±0.401	15.72±0.304	61.52
9	S-9	17.88±0.290	15.96±0.331	12.43±0.490	17.88±0.290	69.98
10	S-10	8.33±0.676	5±0.541	1.84±0.222	8.33±0.676	32.6
11	K-1	12.11±0.378	9.12±0.304	3.01±0.444	12.11±0.378	47.39
12	K-2	11.04±0.449	7.32±0.403	1.4±0.416	11.04±0.449	43.2
13	K-3	1.14±0.274	5.32±0.372	11.14±0.385	11.14±0.385	43.6
14	K-4	9.94±0.449	14.9±0.322	12.25±0.519	14.9±0.322	58.31
15	K-5	12.62±0.635	8.37±0.384	3.15±0.620	12.62±0.635	49.37
16	MTCC-170	20.32±0.385	14.9±0.322	10.09±0.453	20.32±0.385	79.53

***Data in mean of three triplicates± S.D**

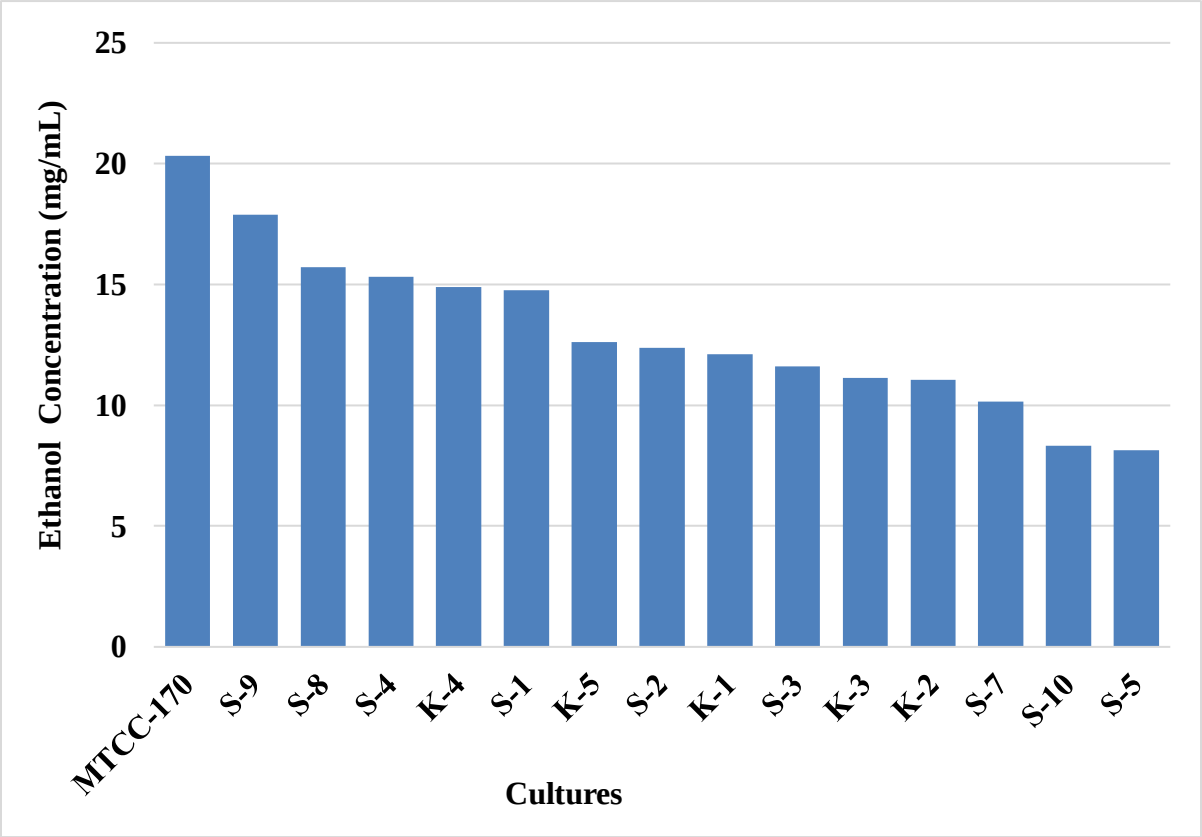


Fig 1. Ethanol concentration of Isolates and MTCC 170 culture in YEPD medium

Table 6: Ethanol content after fermentation of S-9 and MTCC 170 culture with rice straw hydrolysate

S.No.	Culture	Ethanol concentration (mg/mL)			Maximum ethanol concentration (mg/mL)	Fermentation efficiency %
		24hr	48hr	72hr		
1	S-9	12.26±0.521	7.373±0.638	2.01±0.956	12.26±0.521	69.5
2	MTCC 170	14.98±0.230	6.38±0.902	1.29±0.465	14.98±0.230	84.9

***Data in mean of three triplicates± S.D**

spectrophotometrically by recording absorbance at 590 nm after 24h, 48h and 72 h of fermentation. The fermentation condition for growth of S-9 isolate were temperature – 25ºC, 30ºC and 35ºC, pH - 4.6, 5.6 and 6.6 and agitation -static, 120rpm and 180rpm (Table 10, Fig. 5).

4.8.1 Effect of Temperature on Ethanol production

During the studies, S-9 culture show maximum ethanol production of 16.9 mg/mL at 24h of fermentation at 30ºC after that the ethanol concentration reduces to 15.35 mg/mL and 11.12 mg/mL at 48h and 72 h respectively. Table 7 and Fig. 2 show the effect of temperature on ethanol production from S-9 isolate on YEPD medium.

4.8.2 Effect of pH on Ethanol production

The final ethanol concentration in the medium is affected by the pH of the medium. More acidic and basic conditions result in a decrease in the yield of ethanol due to retarded cell growth and metabolic pathways in yeast (Taherzadeh *et al.*, 2007). Optimum pH for the ethanol production by S-9 isolate was 5.6 with the ethanol concentration of 16.96mg/mL after 24 h of fermentation and having the fermentation efficiency of 66.37% (Table 8, Fig. 3).

4.8.3 Effect of Agitation on Ethanol production

Maximum ethanol production of 15.77mg/mL was estimated during fermentation in static conditions. Agitation causes a reduction in the production of ethanol which is due to the increase in cell density. Maximum ethanol production was estimated to be 15.69 mg/mL and 14.75 mg/mL when fermentation occur in shaking conditions at 120 rpm and 180 rpm respectively after 24 h (Table 9, Fig. 4).

4.9 Quality parameters

S-9 isolate show the Ethanol concentration of 12.26 mg/mL, Fermentation efficiency of 69.5%. 50.7% of sugar conversion occur during the fermentation process and volumetric productivity of 0.1702 g/L/h. Ethanol content per gram of sugar utilized was estimated to be 0.168g/g. MTCC 170 culture show a higher ethanol concentration of 14.98 mg/mL. Fermentation of hydrolysate of rice straw occur with fermentation efficiency of 84.9% and 67.09% of sugar consumption was estimated (Table 11).

Table 7: Effect of temperature on production of Ethanol from S-9 Culture on YEPD media

S.No.	Tempera-ture	Ethanol concentration (mg/mL)			Maximum Ethanol concentration (mg/mL)	Fermentation efficiency %
		24hr	48hr	72hr		
1	**25°C**	14.67±0.235	11.19±0.322	6.7±0.654	14.67±0.235	57.41
2	**30°C**	16.9±0.562	15.35±0.385	11.12±0.235	16.6±0.562	66.17
3	**35°C**	15.03±0.225	11.2±0.449	4.89±0.433	15.03±0.225	58.85

***Data in mean of three triplicates± S.D**

Table 8: Effect of pH on production of Ethanol from S-9 culture on YEPD media

S.No.	pH	Ethanol concentration (mg/mL)			Maximum ethanol concentration (mg/mL)	Fermentation efficiency %
		24hr	48hr	72hr		
1	**4.6**	13.9±0.455	10.41±0.680	4.48±0.708	13.9±0.455	54.5
2	**5.6**	16.96±0.449	11.56±0.290	7.41±0.635	16.96±0.449	66.37
3	**6.6**	13.41±0.449	9.62±0.680	4.78±0.482	13.41±0.449	52.48

***Data in mean of three triplicates± S.D**

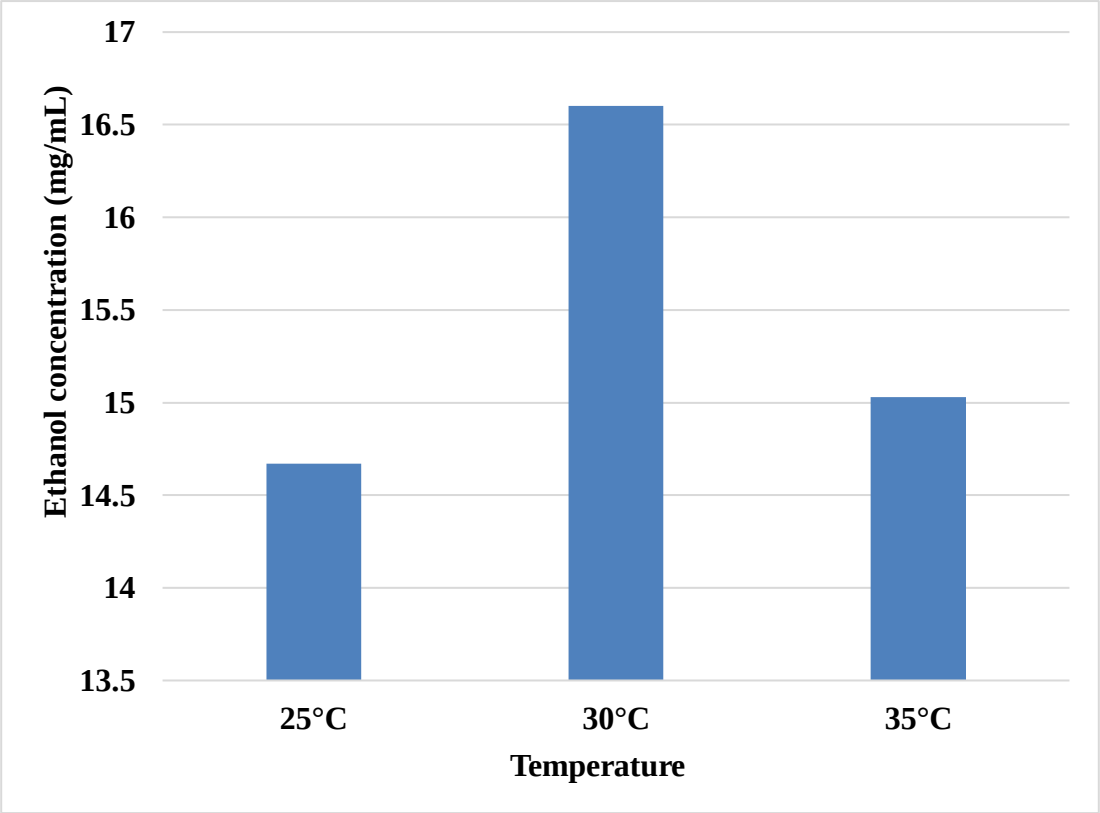


Fig. 2. Effect on Temperature on Ethanol production by S-9 Culture on YEPD media

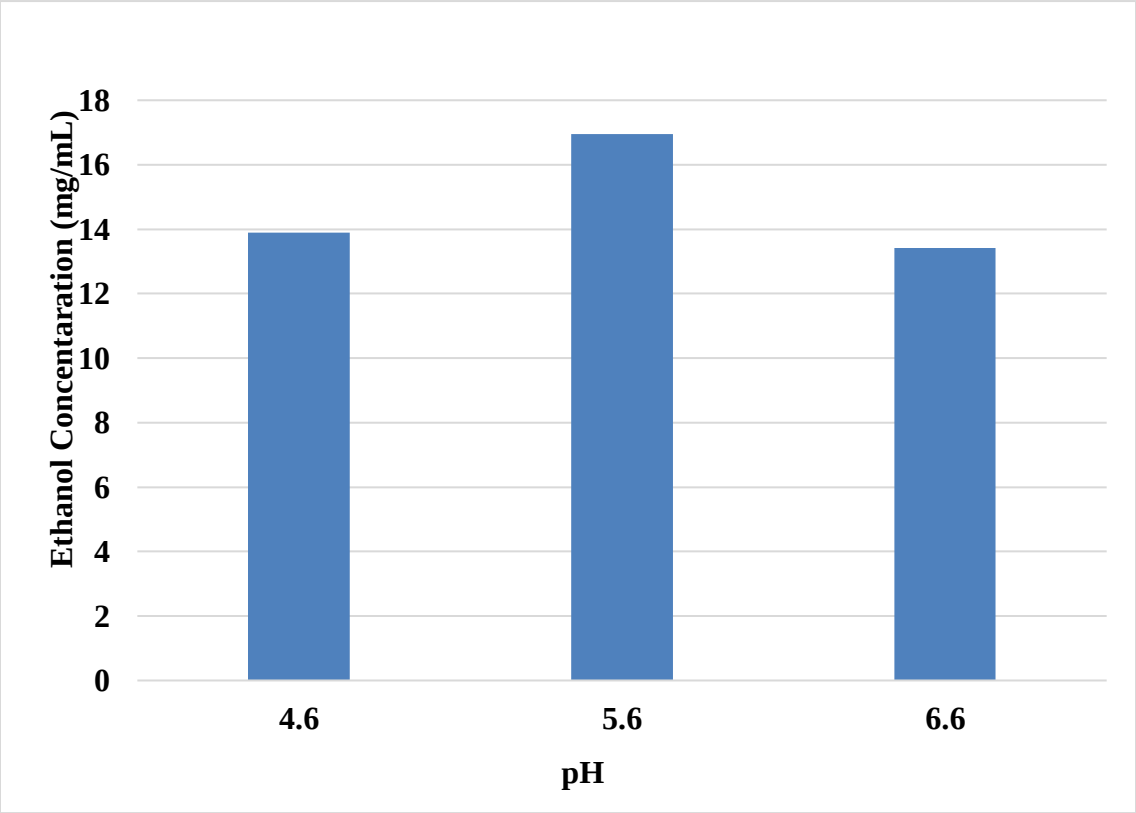


Fig. 3: Effect of pH on Ethanol production by S-9 Culture on YEPD media

Table 9: Effect of agitation on production of ethanol from S-9 culture on YEPD medium

S.No.	Agitation	Ethanol concentration (mg/mL)			Maximum ethanol concentration (mg/mL)	Fermentation efficiency %
		24hr	48hr	72hr		
1	Static conditions	15.77±0.634	11.56±0.612	6.95±0.323	15.77±0.634	61.7
2	120 rpm	15.69±0.806	8.75±0.980	4.46±0.484	15.69±0.806	61.43
3	180 rpm	14.75±0.449	10.19±0.455	5.12±0.819	14.75±0.449	57.72

***Data in mean of three triplicates± S.D**

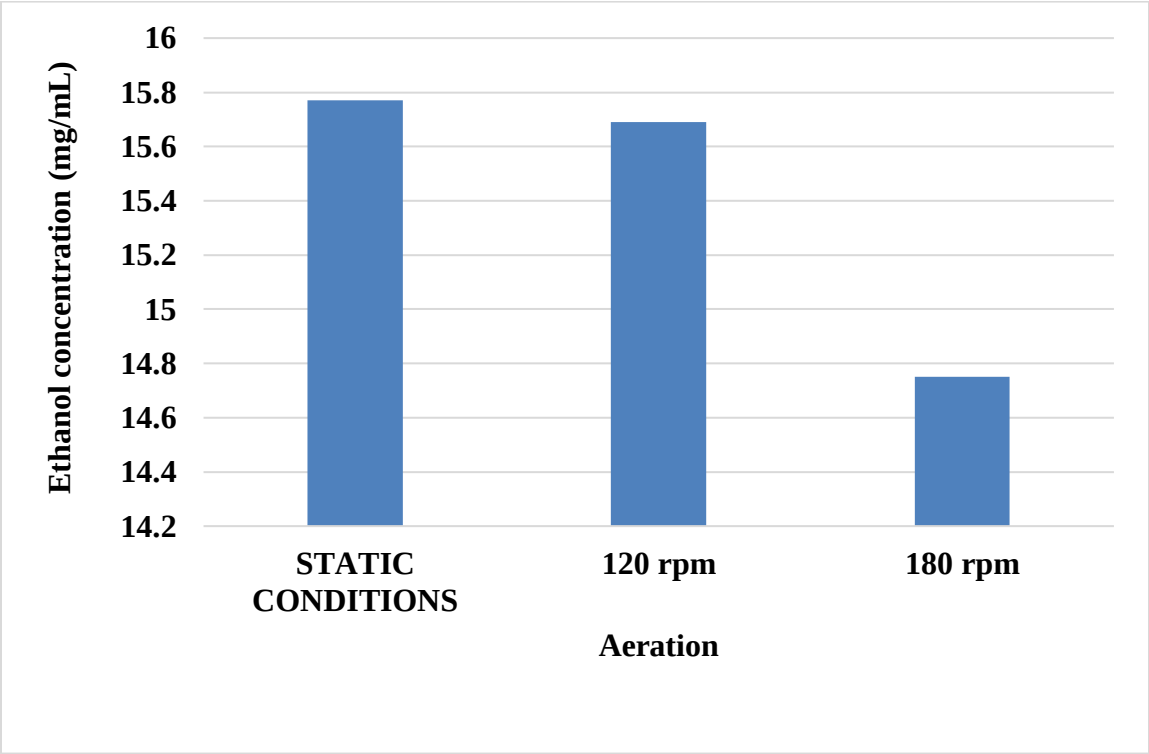


Fig. 4: Effect of Agitation on Ethanol production by S-9 Culture on YEPD media

Table 10: Effect of Temperature, pH and Agitation on Ethanol production by S-9 isolate on YEPD media

S.No.	Optimized conditions		Maximum ethanol concentration (mg/mL)	Fermentation efficiency %
1	**Temperature**	**25°C**	14.67±0.235	57.41
		30°C	16.9±0.562	66.17
		35°C	15.03±0.225	58.85
2	**pH**	**4.6**	13.90±0.455	54.5
		5.6	16.96±0.449	66.37
		6.6	13.41±0.449	52.48
3	**Agitation**	**Static**	15.77±0.634	61.74
		120 rpm	15.69±0.806	61.43
		180 rpm	14.75±0.449	57.72

***Data in mean of three triplicates± S.D**

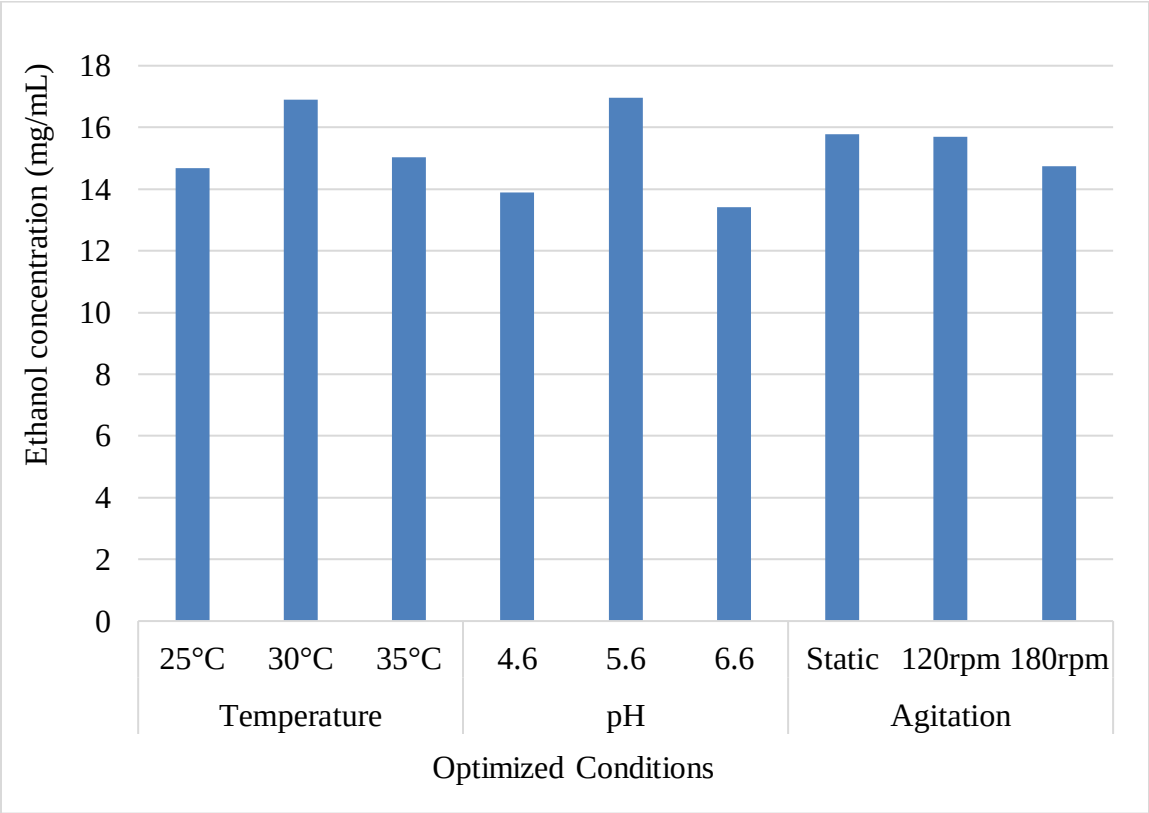


Fig. 5: Effect of Temperature, pH and Agitation on Ethanol production by S-9 isolate on YEPD media

Table 11: Ethanol production during the fermentation of pretreated hydrolysate

Culture	Ethanol concentration (mg/mL)	Fermentation efficiency %	Q_p (g/l/h)	$Y_{g/s}$ (g/g)	S_c%
S-9	12.26±0.521	69.5	0.1702	0.168	50.7
MTCC-170	14.98±0.230	84.9	0.208	0.158	66.36

Q_p volumetric productivity, $Y_{g/s}$ Ethanol content per gram of sugar utilized, S_C Sugar consumption%

***Data in mean of three triplicates± S.D**

Chapter-5

Discussion

CHAPTER 5

DISCUSSION

In the present study, fruit samples and kaladi samples were collected from local market of Jammu and Udhampur district respectively. 15 yeast strains were isolated from Apple, Papaya, Sugarcane, Grapes, Orange, Pineapple and Kaladi samples. Nasir *et al.*, 2017 have isolated *Saccharomyces cerevisiae* from orange and pineapple for bioethanol production. The isolation of *Saccharomyces cerevisiae* from fruit samples has been reported by various workers (Balakumar *et al.*, 2001; Boudjema *et al.*, 2016; Sabate *et al.*, 2002; lee, 2013; Cha *et al.*, 2008, Zin Yu *et al.*, 2018).

Potential yeast isolates were identified on the basis of morphological and microscopic characteristics which were similar to that of MTCC 170 culture. Morphological characters of our culture show smooth whitish, creamish and entire colony on YEPD medium. The cell morphology of our isolated cultures shows ovoid and elongated cells. Our efforts are backed up by the work of Nasir *et al.*, 2017.

Isolates were screened for the bioethanol production on the basis of color change of the screening medium. Nasir *et al.*, (2017) also identified the isolates on the basis of color change of the screening medium. They suggested that the color change of the medium is due to the acid produced and gas evolved during fermentation. The quantification of selected isolates was done spectrophotometrically which revealed that S-9 isolate show maximum ethanol concentration of 17.88 mg/mL at 24 h of fermentation on YEPD medium. Potential yeast strains studies revealed that isolated *Saccharomyces cerevisiae* strain produce maximum ethanol concentration of 16.96 mg/mL at 5.6 pH on YEPD medium.

Rice straw is a potential lignocellulosic material for the production of bioethanol. It is composed of high concentration of cellulose and hemicellulose due to which it can be converted into fermentable sugars. Pretreatment aims to expose the cell wall materials for enzymatic degradation, enhance the substrate's surface area and porosity, diminish cellulose crystallinity, and alter the heterogeneous structure of cellulosic materials (Gadde *et al*., 2009). Rice straw was pretreated by alkaline, acidic and enzymatic hydrolysis. Fermentation of sugar hydrolysate of rice straw by S-9 isolate produces 12.26 mg/mL of ethanol at 24h of fermentation, Fermentation efficiency of 69.5%. 50.7% of sugar conversion occur during the fermentation process

and volumetric productivity of 0.1702 g/L/h. Ethanol content per gram of sugar utilized was estimated to be 0.168 g/g.

In our present study, optimization of fermentation condition for growth of S-9 isolate were temperature – 25ºC, 30ºC and 35ºC, pH - 4.6, 5.6 and 6.6 and agitation - static, 120rpm and 180rpm.

Optimum pH for the ethanol production by S-9 isolate was 5.6 with the ethanol concentration of 16.96 mg/mL after 24 h of fermentation and having the fermentation efficiency of 66.37%. Our results are in accordance with that of Narendranath and Power, (2005). They reported that, *Saccharomyces cerevisiae* in general is an acidophilic organism and thus grows better under acidic conditions. There are many enzymes functioning within the yeast cell during growth and metabolism. Each enzyme works best at its optimal pH, which is acidic because of the acidophilic nature of the yeast itself. When the extracellular pH deviates from the optimal level, the yeast cell needs to invest energy to either pump in or pump out hydrogen ions in order to maintain the optimal intracellular pH (Narendranath *et al.*, 2001). If the extracellular pH deviates too much from the optimal range, it may become too difficult for the cell to maintain constant intracellular pH, and the enzymes may not function normally. If the enzymes are deactivated, the yeast cell will not be able to grow and make ethanol efficiently. The optimum temperature for the ethanol production for S-9 isolate appears to be 30ºC with maximum ethanol value of 16.90 mg/mL. Maurice, (2011) suggested that the yeasts at 30ºC are under minimal stress and are not inhibited by the produced ethanol. In addition, at 35°C, the ethanol yield decreases with an increase of fermentation temperature. Maximum ethanol production of 15.77 mg/mL was observed during fermentation in static conditions. Agitation causes a reduction in the production of ethanol which is due to the increase in cell density. Maximum ethanol production was estimated to be 15.69 mg/mL and 14.75 mg/mL when fermentation occur in shaking conditions at 120rpm and 180rpm respectively after 24 h.

Chapter-6

Summary and Conclusion

CHAPTER 6

SUMMARY AND CONCLUSION

The present study entitled 'Studies on bioethanol production from rice straw by *Saccharomyces cerevisiae*' was conducted at Department of Microbiology SKUAST Jammu with a view to study the bioethanol producing property of isolated strain of *Saccharomyces cerevisiae* using Rice straw as a substrate and also to optimize the fermentation parameters i.e., temperature, pH and agitation to enhance the bioethanol production. In the present study, pretreated rice straw was subjected to enzymatic hydrolysis followed by fermentation of the hydrolysate by *Saccharomyces cerevisiae*.

In the present study we have collected fruit and kaladi sample from the local inhabitants of Jammu and Udhampur district. A total of 15 *Saccharomyces cerevisiae* cultures which include two from Pineapple, two from Grapes, one from orange, one from papaya, one from apple, three from sugarcane and five from kaladi sample were obtained by isolation and purification on YMA media. Characterisation was done by morphological and microscopic studies. Among them, 14 strains were selected for their ability to produce ethanol in the screening media. Further, S-9 culture was chosen due to its highest bioethanol producing ability on YEPD medium. Rice straw was subjected to alkaline, acidic and enzymatic pretreatment in order to break down the rigidity of lignocellulosic biomass so that the lignin, hemicellulose, and cellulose molecules would be exposed, allowing the yeast to access fermentable sugars. Ethanol Fermentation of the total Rice Straw Hydrolysate obtained after pretreatments were performed using S-9 culture. In the present study, we have optimized the fermentation parameters i.e., temperature, pH and agitation for the growth of S-9 culture on YEPD media. S-9 culture shows the maximum efficiency for bioethanol production at temperature of 30ºC, pH of 5.6 and under static conditions. This study revealed that native yeast isolates from local fruits and kaladi could be used to devise bioethanol production technology from rice straw fuel, presently considered as menace by paddy farmers. This can contribute to meet demand of bioethanol by any developing nation.

Following are the conclusions drawn from this novel study:

- A total of 15 cultures were isolated from fruit and kaladi samples collected from local market of Jammu and Udhampur district.
- Microscopic and morphological characteristics verified that the selected isolates were *Saccharomyces cerevisiae.*
- Out of 15 isolates, 14 show a positive change in the color of screening medium indicating the production of ethanol.
- S-9 culture was further selected due to its highest bioethanol producing ability of 17.88 mg/mL at 24 h of fermentation on YEPD medium and hence chosen for the fermentation of rice straw hydrolysate.
- Rice straw was collected from Chatha research farm and subjected to alkaline and acidic pretreatment which was followed by enzymatic hydrolysis.
- Ethanol Fermentation of the total Rice Straw Hydrolysate obtained after pretreatments using S-9 culture revealed ethanol concentration of 12.26 mg/mL at 24h of fermentation, Fermentation efficiency of 69.5%, 50.7% of sugar conversion occur during the fermentation process and volumetric productivity of 0.1702 g/L/h. Ethanol content per gram of sugar utilized was estimated to be 0.168g/g.
- Further, S-9 culture show the maximum efficiency for bioethanol production on YEPD medium at temperature of 30°C, pH of 5.6 and under static conditions.

Thus, S-9 isolate has potential for bioethanol production from rice straw. This yeast culture can be projected as potential ethanol producing candidate for culture improvement that can further be explored for bioethanol industry.

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Appendix

Appendix-I

YMA

Composition		**g/L**
Yeast extract	:	1.000
Mannitol	:	10.000
Dipotassium phosphate	:	0.500
Magnesium sulphate	:	0.200
Sodium chloride	:	0.100
Congo red	:	0.025
Agar	:	20.000
Final pH	:	6.8±0.2

YEPD

Composition		**g/L**
Peptic digest of animal tissue	:	20.000
Yeast extract	:	10.000
Dextrose	:	20.000
Agar	:	15.000
Final pH	:	6.5±0.2

Vita

VITA

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OGPA/ %	:	8.53
Title of Master's Thesis	:	**"Studies on bioethanol production from rice straw by *Saccharomyces cerevisiae*"**

CERTIFICATE-IV

Certified that all the necessary corrections as suggested by the external examiner and the advisory committee have been duly incorporated in the thesis entitled **"Studies on bioethanol production from rice straw by *Saccharomyces cerevisiae*"** submitted by **Miss Neeru Choudhary**, Registration No. **J-19-MBS-38.**

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