OENOLOGICAL CHARACTERIZATION OF TRADITIONAL STARTER CULTURE USED IN THE PRODUCTION OF ALCOHOLIC BEVERAGES

Enrollement No- 111558

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In

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CERTIFICATE

This is to certify that the work presented in this dissertation entitled **"Oenological characterization of traditional starter culture used in the production of alcoholic beverages"** was carried out by **Ms Shivani Bhargove** at Department of Biotechnology and Bioinformatics, Jaypee University of Information Technology, Waknaghat (Solan) under my supervision towards the fulfillment of her Bachelor of Technology in Biotechnology. It is also certified that no part of this dissertation has been submitted elsewhere for the award of any degree or diploma.

Signature of Supervisor

Name of superervisor- Dr. Gunjan Goel

Designation

Date

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I owe a great thanks to many people who have been helping and supporting me during this project.

My deepest thanks to Dr. Gunjan Goel, for guiding and correcting me at every step of my work with attention and care. He has taken pain to go through the project and made necessary corrections as and when needed.

I would also thank Prof R. S. Chuahan, Head of the department, my Institution and faculty members without whom this project would have been a distant reality. I also extend my heart full thanks to my family members and well-wishers.

I would also acknowledge the cooperation provided by the non-teaching staff, especially Mr. Baleshwar, for his guidance and friendly cooperation. I would also like to thank my friends. A tremendous amount of thanks goes to my parents.

At last but not least, I would like to thank God for giving me life and all the blessings and care, I have received along these years.

Signature of the student

Name of Student Shivani Bhargove

Date

SUMMARY

Malera is a traditional culture prepared by the people of Himachal Pradesh. Malera culture is reported to have a mixture of starters with yeast as a dominant microorganism. The objective of this study was to characterize the yeast starter present in Malera and to compare the malera starter with the brewer's yeast oenological characteristics. The tests like ethanol tolerance test, temperature tolerance, osmo tolerance, flocculation ability and tolerance to different pH levels were conducted. In the second part of the experiments, litchi wine was prepared from both the starters. Real Litchi juice is taken from the market and was used as a substrate at a brix of 24 for the production of ethanol along with 2% of di-ammonium phosphate. The juice was inoculated with both starters @ **1.5% and incubated** at 30^0C for 15 days. A sample was withdrawn at a regular interval to estimate the ethanol content and residual sugar content. From the initial comparative data obtained, the Malera yeast has high ethanol, pH and osmo tolerance. The yeast starter also resulted in better flocculation than the standard brewer's strain. When inoculated in the litchi juice, the malera starter was observed to have faster metabolic activity in terms of ethanol production as well as in reduction of reducing sugar content. From the results obtained, it is concluded that malera culture can be used at larger scale for the production of alcoholic beverages at lower cost of production.

Signature of Student	Signature of Supervisor
Name	Name
Date	Date

LIST OF FIGURES

LIST OF ABBREVIATIONS

ABBREVIATIONS	EXPANDED FORMS
LAB	Lactic Acid Bacteria
◦**C**	degree Celsius
%	Percentage
L	Liter
dw	distilled water
fig	Figure
g	Gram
g/L	Grams per liter
h	Hours
mg	Milligram
Mg/ml	Milligram per milliliter
µg	Microgram
µg/l	Microgram per liter
OD	optical density
w/v	Weight by volume
sec	Seconds
nm	Nanometer
DNS	Di-nitro salicylic acid
YPD	yeast peptone dextrose
YPDA	Yeast peptone dextrose agar
TSS	Total soluble sugars

CHAPTER 1

INTRODUCTION

Enology, a word comes from the Greek words for wine and study, is the term used to describe the science of winemaking.(Amerine et al.,1978).This science has its roots in pre-historic times, as the effects of alcohol were probably discovered when rotten fruit was consumed and found to have an intoxicating effect, which was considered as pleasant.

Fermentation is a complex process, affected by various parameters ,which have an impact on the wine so formed. The factors influencing fermentation are- (Joshi et al.,1999)

a. Carbon/energy sources: This means the supply of soluble sugars in the juice. *Saccharomyces cerevisiae* is capable of fermenting only a few simple sugars, and of those found in wine , only glucose and fructose are fermentable. The high sugar content may cause difficulty, initially due to osmalarity of the juice and later , due to the combined effects of ethanol and the osmolarity of the juice.
b. Nitrogen content: Some fruits like grapes have ample ammonium or amino nitrogen to complete the fermentation whereas other fruits require the addition of ammonium salts which indirectly affects the aromatic character of wine.
c. Lipids: They play an important role in yeasts, including nutrient storage and regulation. However, relative to fermentation their main significance involves cell membrane function.
d. Alcohol content: The increasing content of alcohol eventually inhibits yeast metabolism even in the presence of fermentable sugars. Ethanol disrupts the transport of sugars across the cell membrane.
e. Temperature: It can influence fermentation by affecting the rate of enzyme action.

Wine industry statistics in India-

- The size of the wine industry in India is estimated at Rs 1,050 crore and has grown at a compounded annual growth rate of 33% between 2003 and 2009.
- Volume of wine consumed in India has grown from 26 lac litres in 2003 to 1.67 crore litres in 2009.
- The wine industry is estimated to achieve 7.2 crore litres of wine consumption by 2020 at a compounded annual growth rate of 18%.
- Growth of the domestic wine industry has provided significant emplyment opportunities to unskilled labourer (Source- Holkar and Jadhav, all India wine producers association, headquarters Worli, Maharashtra)

The efficiency of yeast strains is determined by their ability to utilize sugar substances, ethanol tolerance capacity, growth at 37^{O}C and alcohol production capacity of yeast strains. The selection of yeast strains depends on their oenological characteristics, such as fermentative rate, tolerance to alcohol , flocculent characteristics, sugar tolerance, higher alcohol production, alcohol yielded and thermal tolerance (Barre, 1980; Benda, 1982; Darriet *et al*., 1988,Dubordieu *et al*., 1988; Duteurtre *et al*., 1990; Valade and Rinville, 1990; Degre,1993; Giudici *et al*., 1993; Zambonelli *et al*., 1994).

Himachal Pradesh is well known for its biodiversity since centuries, positioned between north 30^{o} 22' 40" to 33 o 12' 20" latitudes and east 75 ^{o} 45' 55" to 79 ^{o} 04' 20" longitudes. This region covers a wide range of mountainous terrain, with the hills falling in the range of 350 to 6975 meters above the sea level. Himachal Pradesh is famous for its abundant natural beauty and bioresources. However, these resources are often exploited. The effort is being made by the government to protect it and some of them have been preserved with the tribal communities of this belt. The microbial characterization of traditionally produced starter cultures of Himachal Pradesh is important to screen the microbial diversity present in the food or beverage that the local inhabitants consume owing to their specific function. These cultures such as *phab*, *murcha*, *dhehli*, *malera*, *khameer*, *balam* are prepared by local communities utilizing native flora from the forests in a particular season (generally September) by carrying out natural fermentation without being aware of the significant role of the microbes involved (Angmo et al.,2014).

A number of traditional fermented products are prepared and consumed in Himachal and the types of traditional fermented products of Himachal are unique and different from

other areas.

Fermented foods, whether from plant or animal origin, are an intricate part of the diet of people in all parts of the world. It is the diversity of raw materials used as substrates, methods of preparation and sensory qualities of finished products that are so astounding as one begins to learn more about the eating habits of various cultures. The preparation of many indigenous or "traditional" fermented foods and beverages remains today as a household art.

S.cerevisiae is also reported as the most studied and biochemically best understood species of the yeast domain. It is best known for its domesticated role in the production of fermented products. This yeast converts hexose sugars to ethanol, CO2, and a variety of compounds including alcohols, esters, aldehydes and acids that contribute to the sensory attributes of the food and beverage (Viljoen BJ et al., 2000).Fermentation of carbohydrates in fruits, grains to ethanol by *S. cerevisiae* is the critical process for a wide range of products (Ceccato-Antonini SR et al.,2004).

Bioprospecting for novel starter cultures is always a need of commercial sector. Therefore keeping the above points in view, this research was planned to characterize the yeast starter from traditional starter known as 'Malera' and to compare the metabolic activities with the standard brewers strain.

CHAPTER 2

REVIEW OF LITERATURE

The term 'fermentation' is derived from the latin verb, fevere, to boil. Fermentation technology is one of the oldest food technologies that have been used for several thousand years as an effective and low cost means for preserving foods and beverages (Borgstrom 1968). Food fermentation is of prime importance in the developing countries where the limitation of resources encourages the use of locally available fermented food products for additional nutrition. These fermented products are more common among people belonging to rural areas, without much awareness about the microflora involved in their production. In the past few years, great empHasis has been given to identify unknown microflora associated with these products. This microflora involves a combination of bacteria, yeast and fungi which have been reported by several workers from various fermented foods viz. kinema (Kim-Bong-Joon 2000), bushera (Muyanja et al. 2003) and togwa (Mugula et al.,2003).

The most important organism associated with fermentation is yeast. Yeasts as a group of micro-organisms have been commercially exploited as a fermentative species to carry out alcoholic fermentation, especially *Saccharomyces cerevisiae*. The importance of this microorganism has urged many scientists to study the factors governing its growth, survival and biological activities in different ecosystems (Heard and Fleet 1985).S. cerevisiae plays a prominent role in controlling the quality and flavor of the final product in wine fermentations (Joshi et al. 2009) and that's why, it has received considerable attention in fermentation industry. To obtain the best strain, knowledge of S. cerevisiae diversity associated with a particular fermented product in a given area, is of prime importance.

2.1 Traditional fermented foods of Himachal Pradesh

In spite of scientific and technological revolution, the art of fermentation practiced by common man has continued, but largely remained confined to the rural and tribal areas due to

(i) high cost or inaccessibility of the industry-made products in remote areas

(ii) taste of the people for the traditional fermented products and

(iii) their sociocultural linkages with such products (Thakur et al. 2004).

Indigenous fermented foods are an intrinsic part of diet of the ethnic tribes in the Himalayan belt of India, being the oldest and most economic methods for biological enrichment of food products by the manipulation of different microbial population (Nehal 2013). Most of the traditional fermented foods are prepared by processes of solid substrate fermentation in which the substrate is allowed to ferment either naturally or by adding starter cultures. Majority of fermented foods and beverages involves filamentous fungi and are produced in East and South-East Asia (Tamang 1998). The traditional fermented foods and beverages form an important constituent of staple diet of the people belonging to the tribal belts of Lahaul & Spiti, Kinnaur, Chamba and rural areas of Kullu, Shimla, Mandi and Kangra districts of Himachal Pradesh (India) where wide range of such type of fermented products are prepared and consumed (Kanwar et al. 2007). Traditional fermented foods are generally nutritious and form the basic components of the diet as staple, adjunct, condiment and beverage, providing calories, proteins, vitamins and minerals to the people (Tamang et al. 1996) Savitri and Bhalla (2007) studied a wide range of traditional foods and beverages unique to tribal and rural belts of Himachal Pradesh (Kinnaur) which constitute a part of staple food consumed during marriages, local festivals and special occasions. Bhatooru, Siddu, Marchu, Seera, Chilra, Manna, Aenkadu, Sepubari, Patande, Doo, Baari, Dosha, Malpude, Babroo, Bedvin Roti, Madrah, Tchati, Churpa, Sura, Chhang, Kinnauri, Angoori, Chulli, Lugri, Arak/Ara, Rak, Chukh and pickles made from different fruits, vegetables and cereals are some of the popular traditional products in rural areas of Himachal Pradesh.

Though the fermentation industries bought in a range of products but the authenticity still lies with the native population and are well documented in Traditional Knowledge Digital Library (2001) under the Traditional Knowledge Act and National Biodiversity Act, 2002.

FIG 1 Various districts of the state Himachal Pradesh

2.2 Screening of culture

Malera is a previously fermented wheat flour dough used for the fermentation of *siddu, marchu, bhaturu, bedvin roti* and *gulgule.* Fermentation in bhatooru significantly enhanced the B vitamin levels especially thiamine, riboflavin and nicotinic acid and essential amino acids viz methionine, pHenylalanine, threonine, lysine and leucine (Savitri et al., 2013).

2.3 Preparation of malera

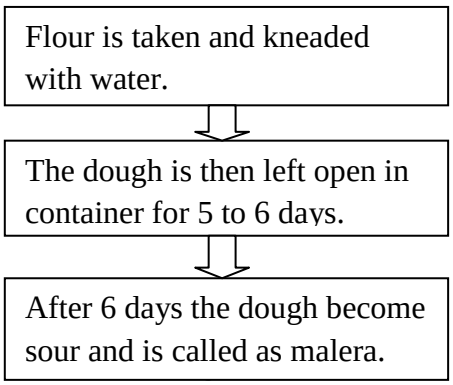


FIG 2 Preparation of malera culture

2.4 Micro organisms reported in culture

Use of microorganisms in preparing foods from locally available plants is a traditional practice since pre-historic times (Ross et al. 2002). A variety of microorganisms are responsible for carrying out fermentation in the fermented products by playing an essential role in bringing out the biochemical changes during fermentation (Basappa and Venkataramu 1994). The microbiological analysis of *malera* revealed that it was a consortium of microorganisms which mainly consisted of lactic acid bacteria and yeast. *Lactobacillus plantarum* (MTCC 8296), *Leuconostoc* sp. And *Saccharomyces cerevisiae* (MTCC 7840) were isolated from different samples of *malera*. The microflora of *malera* depends on flour, water used for dough preparation, utensils used, prevailing hygienic conditions as well as various parameters of the fermentation. The most relevant bacteria isolated from sourdough belonged to the genus *Lactobacillus* (Stolz 2003). Various yeast strains have also been isolated from spontaneous sourdough fermentations such as *Saccharomyces cerevisiae* and *Pichia satoi* (Beech and Davenport 1971). There have been several reports (Okada et al. 1992; Oura et al. 1982; Spicher 1984; Spicher and Schroder 1978, 1980) of lactobacilli occurring among the dominant microbial population in sourdough where they contribute to dough fermentation. *Lactobacillus* species are widely distributed in various fermented foods, dairy products and plant and animal materials (Cai et al. 1999).In earlier papers reported by Thakur et al. (2004) used culture dependent method for characterization of *malera* reported *Saccharomyces cerevisiae* as the major species playing role in fermentation. Various morphological and biochemical assays were carried out for starter inoculums collected from Lahaul and Spiti (H.P.)

(Kanwar et al., 2007, 2011). 16S rRNA studies (Sharma et al.,2013) and morphological studies (Sharma et al.,2014) were also being carried out for fermented beverages and foods such as *chhang, lugri, seera, sepubari, bhaturu, gulgule* reporting the presence of *Lactobacillus sp.* and *Saccharomyces cerevisiae*. Several studies were also carried out to estimate the ethanol content of various beverages. However, the oenological characterization of *malera* culture has not been done. Ethanol tolerance, sugar tolerance and invertase activities are some of the important properties for use in industrial ethanol production (Jimenez J et al., 1986). A large number of bacteria and yeast were isolated from fermented dough samples of *bhatooru* fermentation at different intervals of time. The microflora of the fermented dough was mainly dominated by yeast (*Saccharomyces cerevisisae*), lactic acid bacteria (*Lactobacillus plantarum*) and *Bacillus* sp. The gas producing *Leuconostoc* sp. also appeared at 4 h of fermentation causing leavening of dough. The source of these organisms might be the ingredients, vessels, and the surroundings followed by rapid multiplication during fermentation. With the progress in fermentation, total microbial count increased from 6×10^4 to 1×10^8 cfu/g decreasing the pH from 5.94 to 4.18. The decrease in the pH prevents the growth of undesirable microorganisms but the desirable microorganisms like yeast, *Leuconostoc* and *Lactobacilli* can very well propagate at this pH. *Saccharomyces cerevisisae* has been reported from various fermented foods and beverages such as *bhalle*, beer, *burukutu*, bourbon whiskey, coffee beans, cider, *merissa*, *fufu*, *tape*, *ogi*, *puto*, *dosa*, *idli*, *papdam*, *kecap*, *lao chao*, *warri*, scotch whiskey, etc. (Padmaja and George 1999; Batra and Millner 1974, 1976; Soni and Sandhu1990). Some species of *Bacillus* and other bacteria such as *Kocuria rhizopHila*, *Pseudomonas synxantha* and*Microbacteriun saperdae* were also found during the initial stages of *bhatooru* fermentation and these organisms gradually disappeared with the progress of fermentation. This may be due to the production of acids and gas from various carbohydrates by lactic acid bacteria thus making the environment unfit for many of the bacterial population initially present.

CHAPTER 3

MATERIALS AND METHODS

3.1 Yeast strains-The strains currently worked on in this project are-

- *Malera* culture is taken from Himalayan belt. The samples of malera starter were collected in sterile containers, transported to the laboratory and kept refrigerated until further processing.
- The standard strain *Saccharomyces cerevisae* was obtained from a local brewery – Minchy's Food Products, Shoghi, Himachal Pradesh, India.

A. **Malera culture**

B. **Brewer's yeast**

Fig 3 Different starters used in the fermentation

Litchi juice- Real Litchi juice was taken from the market and was used as a substrate wine production

Chemicals –

a) Composition of media for maintenance and preservation of yeast cultures (YEPD Agar):

- Yeast extract powder (HIMEDIA)
- Peptone, bacteriological (HIMEDIA)
- Dextrose anhydrous powder (C6H12O6) (Fisher Scientific)
- Agar - Agar purified (for microbiology) (MERCK)
- Distilled water

b) Staining of strains

Crystal violet (MERCK)

c) Nutrients for yeast in the fermentation process include :

- Ammonium phosphate dibasic (di Ammonium hydrogen ortho-phosphate)

extrapure AR (SISCO RESEARCH LABORATORIES PVT. LTD)

d) Ethanol (MERCK)

e) Chemicals required for DNS test :

- 3,5- di- nitro salicylic acid (B. Genei)
- Sodium potassium tartarate (MERCK)
- Sodium hydroxide pellets purified (MERCK)

f) Chemicals required for qualitative estimation of ethanol:

- Potassium dichromate
- Sulfuric acid

3.2 METHODS :

1. Maintenance and preservation of yeast culture

(Yeast Extract Peptone Dextrose Agar) YEPDA media was prepared to provide a suitable environment for growth of *Saccharomyces cerevisae* strains. The media was prepared by adding the following: Yeast extract powder (1.0gm), Dextrose (2.0gm), Peptone (2.0gm) and Distilled water (100ml). A 0.1g of sample was inoculated on to YEPD broth medium. The flask were incubated at 30^0 C for 3 days. After 3 days of incubation, streaking was being done on plates containing YEPGA media. The plates were examined for colony formation and colonies suggestive of yeasts were preliminary identified by microscopic examination of smears for studying cell morphology and budding characteristics. For obtaining pure culture, a loop full of colony from above plates was inoculated in YEPD broth and also 0.1 ml of each culture was streaked onto YEPD agar plates. The plates were incubated at 30^0C for 3 days.

A.MALERA CULTURE	B.BREWER'S YEAST

Fig 4 Pure cultures of yeast starters

2. Staining

Staining was a common technique used to differentiate microbes based on their different cell wall constituents, structures, etc. Staining involved three processes: staining with a water-soluble dye called crystal violet, de colorization, and fixing the stain. The slide of each of strain colony sample was made. The sample was heated to the slide by carefully passing the slide with a drop or small piece of sample on it through a Bunsen burner three times. Then, the primary stain (crystal violet) was added to the sample/slide and incubated for 1 minute. The slide was rinsed with a gentle stream of water for 5 seconds to remove unbound crystal violet. Then, added Gram's iodine for 1 minute- this is a mordant, or an agent that fixes the crystal violet to the cell wall. Washed the slide with a gentle stream of water for 5 seconds, and then viewed under a microscope.

3. Temperature tolerance

The isolated yeast strains and reference strain *Saccharomyces cerevisiae* MTCC 170 were inoculated in reagent bottles containing YEPD media for checking the effect of temperature on the growth. The bottles were incubated at 30^0C and 37^0C for 72 hrs. At 24 hr , 48 hr and 72 hr sample was taken in an eppendorf and absorption was measured at 600nm to check the growth.

4. Ethanol tolerance

Ethanol tolerance of culture and reference yeast strains was tested by inoculating 5% broth culture of each strain in liquid YEPD media supplemented with 5% , 7% and 10% ethanol.

After inoculation, flasks were incubated at 30^0C for 48 hrs. Samples were taken at 24 hrs, 48hrs and 72hrs.Optical density of the samples was recorded at 600 nm on U.V-Visible Spectrophotometer (ELICO, India) to check the growth. All experiments were carried out in triplicates and mean values were considered.**(Iara P. Machado et al., 2006)**

5. Flocculation test-The reference strain and the isolated starter culture inoculate were inoculated in 10 mL of liquid YEPD and incubated at 30^oC for 72 hours. After

incubation, the tubes were agitated for the visualization of flocculation.(Danilo G. Moriel et al.,2006)

6. Sugar tolerance (Osmotolerance)

The reference yeast strain and the isolated culture were tested for their osmotolerance by testing their growth in YEPD broth (in triplicates) containing 5, 10, 15, 20% glucose concentration. Actively growing yeast cultures (10%) were inoculated in triplicates and flasks were incubated at 30^0C. Samples were taken at 24 hrs, 48 hrs and 72 hrs. Optical density was recorded at 600 nm on U.V-Visible SpectropHotometer (ELICO, India) to check the growth.(Mir Naiman Ali et al ., 2014).

7. pH tolerance test

The reference strain and the isolated culture were tested for their pH tolerance test by checking their growth in YEPD broth (in triplicates) having pH 3, 4, 5, 6 maintained in reagent bottles. Samples were inoculated and the bottles were incubated at 30^0C. Samples were taken at 24 hrs, 48 hrs and 72 hrs. Optical density was recorded at 600nm on U.V-Visible Spectrophotometer (ELICO, India) to check the growth.

8. Setting up fermentation

a) Collection of litchi juice

Real Litchi juice is taken from the market and was used as a substrate for the production of ethanol .

b) Setting up the required brix

Actual sugar content of litchi juice was measured which comes out to be 15 brix. Sucrose was being added to the juice till the sugar content reached to 24 brix.

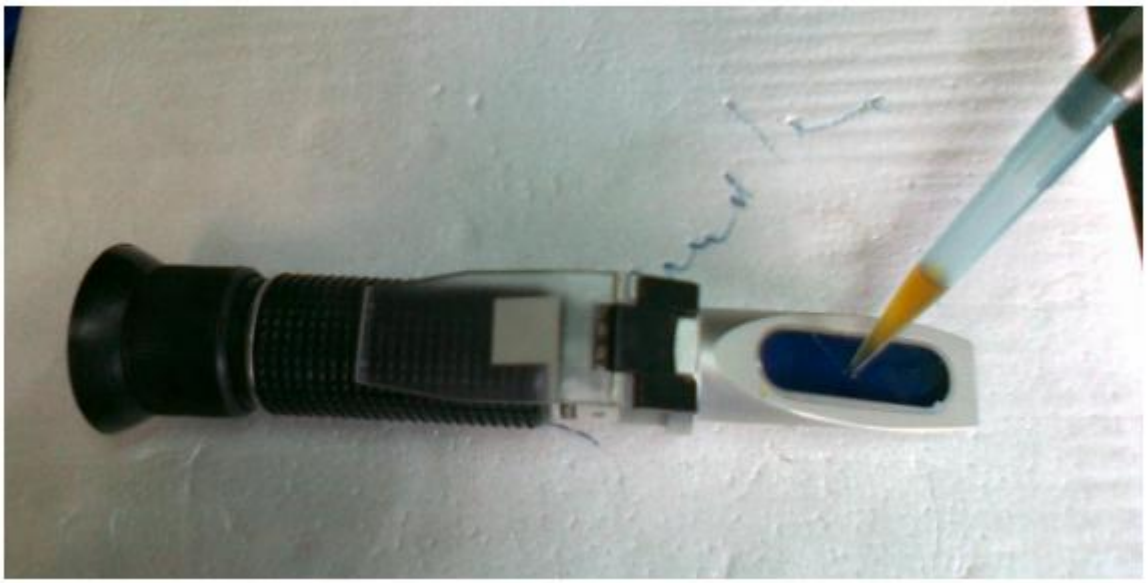


FIG 5 How to use a hand held refractometer

c) Addition of di-ammonium phosphate (DAP)

.2%w/v DAP was added to the litchi juice to provide nitrogen source for the strains to ferment.

d) Inoculation of culture in the juice

1.5% of the reference strain and the cultured strain was added to the reagent bottles.

e) Activation of the culture

The reagent bottles were kept in water bath at 30^0C for the activation of culture.

f) Incubation

The reagent bottles were incubated at 25^0C – 30^OC for 7 days.

9. Check for ethanol production

The reference strain and the cultured strain were tested for their ethanol production qualitatively by potassium dichromate test.

a) Preparation of potassium dichromate

To 125 ml of distilled water in a 500ml conical flask, 70ml of sulfuric acid was added with a constant mixing. A 0.75 gm of potassium dichromate was added to it.

b) Qualitative estimation of ethanol

A 2 ml of sample was taken and to each sample one drop of potassium dichromate solution was added.

10. Check for ethanol yield

a) Qualitative estimation of ethanol was done with the help of potassium dichromate test. The sample was taken and 2 drops of potassium dichromate solution were added to the sample. The tubes were observed for color change.

b)Quantitative estimation of ethanol was done with the help of alcohol-meter. Sample was taken in a measuring cylinder and alcoholmeter is put in the cylinder. The reading of alcohol-meter will give the alcohol content in percentage.

FIG 6 Pictorial representation of alcohol-meter

11. Sugar estimation using DNS Test

The test by miller (Miller et al.,1959) for calculation sugar estimation by DNS test indicates the end of the fermentation process when the amount of sugar available for fermentation becomes constant.

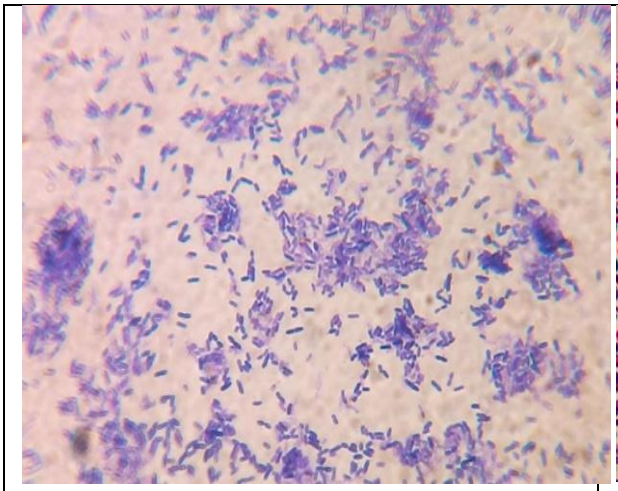
DNS reagent was prepared by adding NaOH-1M (4GM), Sodium potassium tartrate (30gm) and DNS (1gm) in 100ml distilled water and stirred for about 10-12 mins using a magnetic stirrer.

A 5ml solution of known concentration of glucose was made in order to make a standard curve. 2ml of DNS reagent was added to 1ml glucose sample of known concentration in a test tube. For the wine samples also, 2ml DNS reagent was added to 1ml of the sample. After cooling to room temperature in a cold water bath, the absorbance at 540nm was recorded with the help of a spectrophotometer. The OD measured at 540 nm was plotted against respective concentrations to get a standard plot.

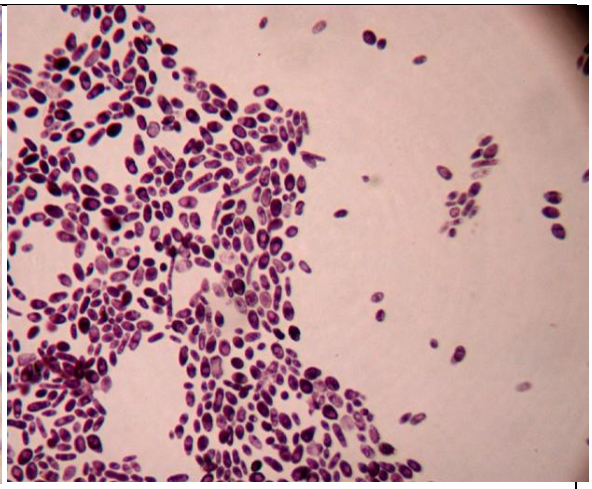
CHAPTER 4

RESULTS AND DISCUSSION

1. Staining of different *Saccharomyces* strains



A.MALERA CULTURE



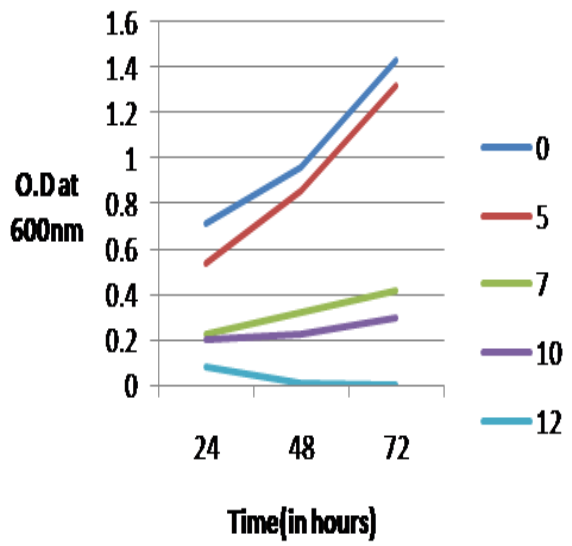
B.BREWER'S YEAST

FIG 7 Simple stain of different yeast strains.

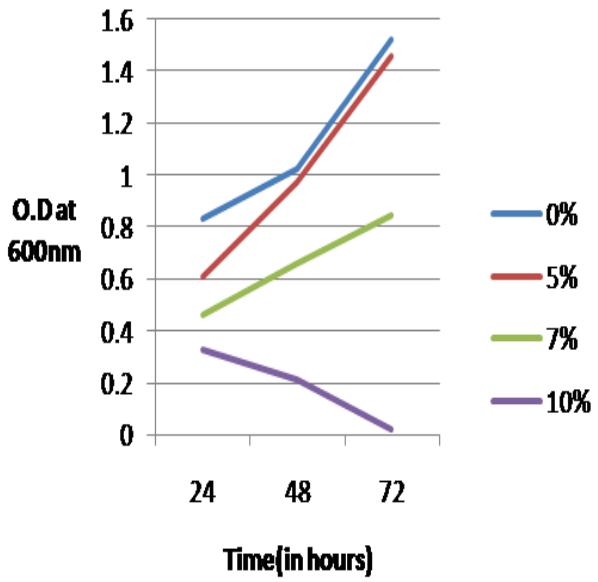
The purple stained cells in malera culture proved the presence of rod shaped bacilli in the culture. Whereas brewer's yeast contained only yeast cells proved by the presence of bud shaped cells. There was no other type of micro organism present in the brewer's yeast.

2. Ethanol tolerance of different strains

By checking the optical density of the strains in a nutrient media containing 0%, 5%, 10%, 15% and 20% ethanol at 24 hrs,48hrs and 72hrs , it was recorded that



A.MALERA CULTURE



B.BREWER'S YEAST

FIG 8 Ethanol tolerance Test for different yeast strains.

It can be inferred from the graph that ethanol tolerance of a strain shows the concentration of alcohol that can inhibit the growth of yeast in a fermentation process.

- Better fermentation needs better and more ethanol tolerant strain.

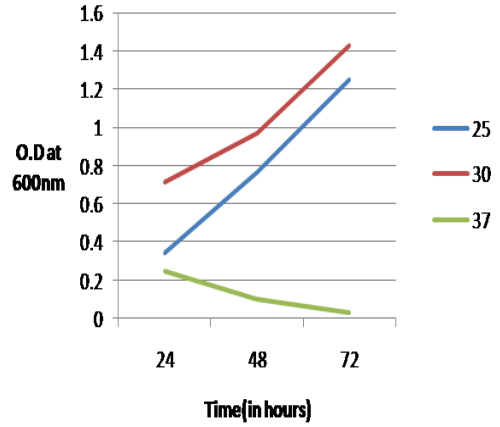
Malera culture had the better ethanol Tolerance than the commercially available *saccharomyces cerevisiae.*

Therefore, Malera can be used to get the following benefits in the fermentation process-

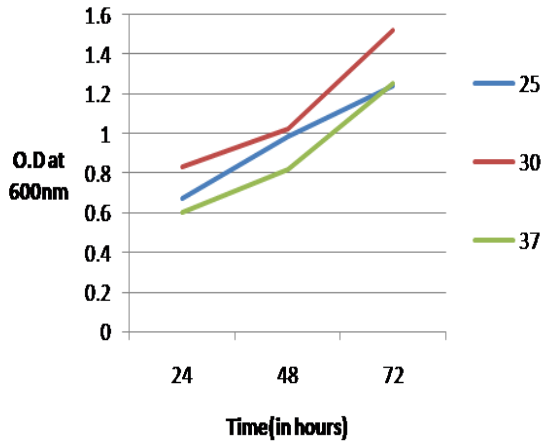
- Conservation of traditional culture.
- Better fermentation capacity.
- Allows more ethanol to be produced per batch.
- Less sensitive to ethanol.
- Decreasing costs and energy consumption.
- Could be incorporated into a large scale industrial fermentation process

3. Temperature tolerance of different strains

By checking the optical density of the strains in a nutrient media maintained at 30 and 37 ^{0}C at 24 hrs,48hrs and 72hrs.



A.MALERA CULTURE



B.BREWER'S YEAST

FIG 9 Temperature tolerance test of different yeast strains

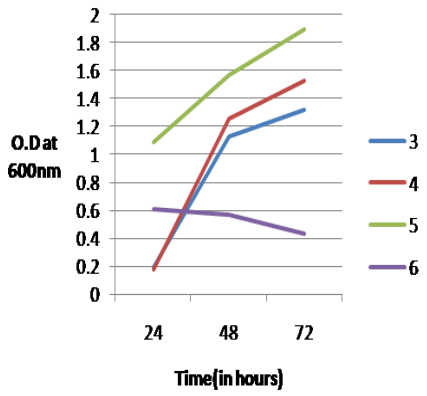
From the results obtained, it can be inferred that

- Both the strains showed maximum growth at 30^{o}C.
- As the temperature rises above 35^{o}C, the growth of the yeast strains affected, complete inhibition of growth was observed for *malera* strains from 37 to 45^{o}C.
- Elevated temperature is problematic in all phases of ethanol production, it is specifically hazardous during the later stages of fermentation. As ethanol accumulates, the optimum temperature range to maximize ethanol growth becomes narrower. Higher temperatures also increase yeast sensitivity to lactic and acetic acids, which causes lower ethanol yields.

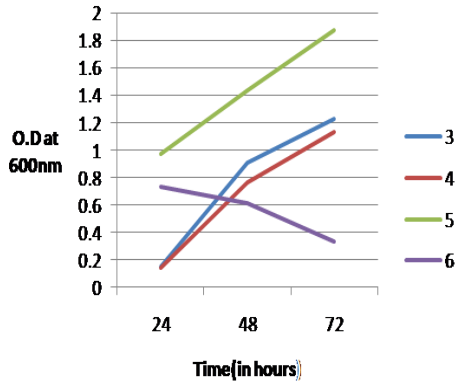
Therefore, it can be said that *malera* culture will not become sensitive to lactic and acetic acid, thus gives better fermentation.

4.pH tolerance of different strains

The starters were grown in YEPD at different pH levels of 3, 4, 5, 6 for 24 hrs,48hrs and 72hrs.



A.MALERA CULTURE



B.BREWER'S YEAST

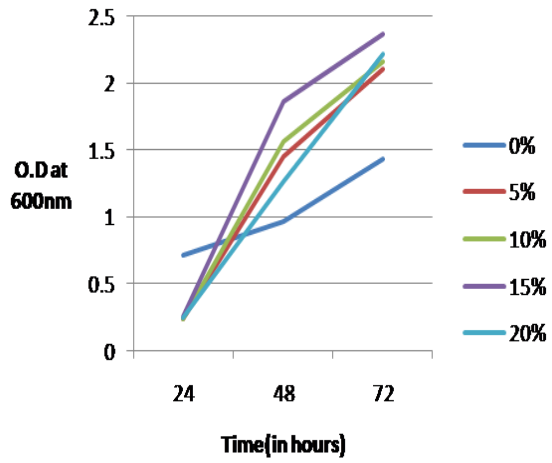
FIG 10 pH tolerance test of different yeast strains

It was inferred that-

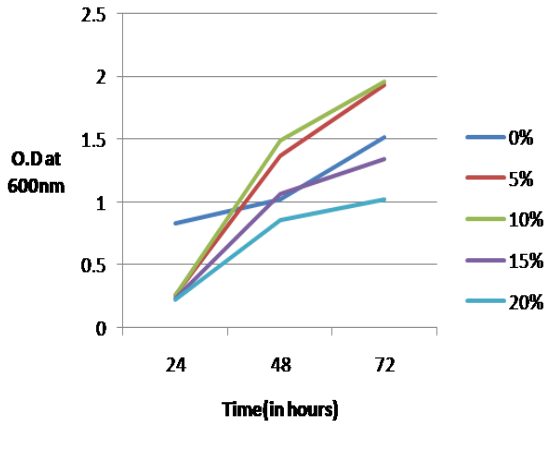
- Acids give wines their characteristic crisp, slightly tart taste as well as help in the growth and vitality of yeast during fermentation and protect the wine from bacteria. Alcohol, sugars, minerals, and other components moderate the sourness of acids and give wines balance.
- Some acids are naturally present in the base ingredients of wines, while others are byproducts of fermentation.
- Increase in pH lowers the acidity of the wines.
- Both *malera* culture and commercially available *saccharomyces cerevisiae* shows maximum growth at pH 5.

5. Sugar tolerance of different strains

The effect of sugar concentration on growth of yeast isolates was studied in order to test the osmotolerance. By checking the optical density of the strains in a nutrient media containing 0%, 5%, 10%, 15% and 20% glucose concentration at 24 hrs,48hrs and 72hrs , it was recorded that



A.MALERA CULTURE



B.BREWER'S YEAST

FIG 11Sugar tolerance test of different yeast strains.

The reference strain of *Saccharomyces cerevisiae* tolerated a sugar concentration of 20% followed by decline in growth whereas malera culture shows no decline in growth at 20%. Therefore, it has better fermentation ability as this is sugar which gets converted to ethanol. Therefore, better is the osmotolerance, more will be the ethanol production.

6. Flocculation test:

The term "flocculation" refers to the tendency to form clumps of yeast called flocs. The flocs (yeast cells) descend to the bottom in the case of bottom-fermenting yeasts or rise with carbon dioxide bubbles to the surface in the case of top-fermenting yeasts. (Smith 2010).The yeasts were also tested for their flocculation abilities. The flocculation is an important characteristic that allows an easy separation of the final product at the end of the fermentation without additional filtration/ centrifugation steps and also allows the utilization of immobilized yeasts on fermentation processes (Stratford, 1992).

	
A. MALERA CULTURE	B.BREWER'S YEAST

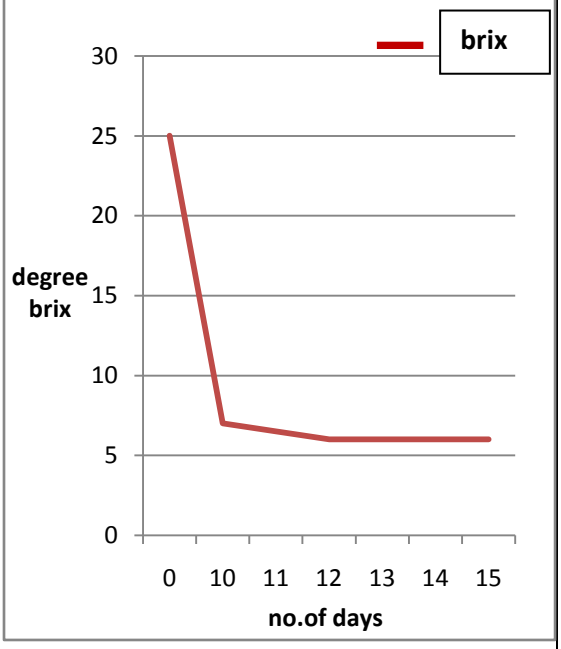
FIG 12 Flocculation test of different yeast strains.

It can be inferred from the figure that the malera culture shows positive test for flocculation. Therefore, it could be a better culture for fermentation as the flocculation ability makes the downstream process and product recovery easy**.**

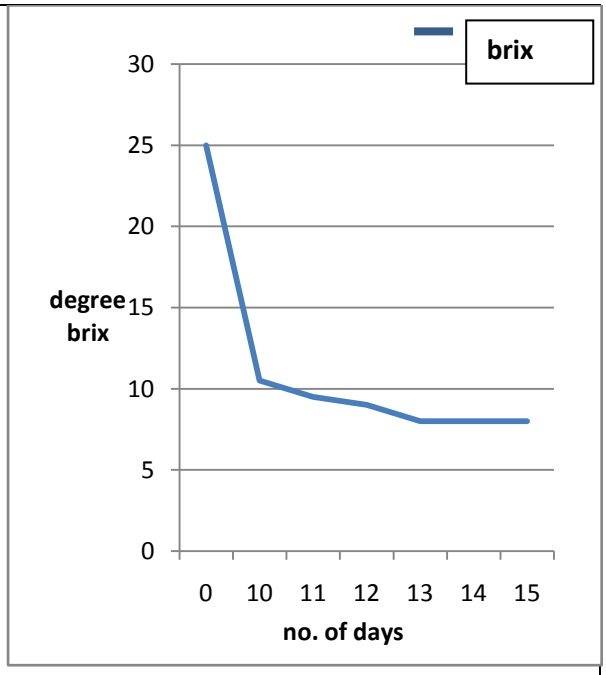
7. ºBrix level estimation

This was used as a measure of the total soluble solids in the juice and was measured with the help of a hand-held refractometer. These soluble solids are primarily sugars; sucrose, fructose, and glucose. Citric acid and minerals in the juice also contribute to the soluble solids. Brix is reported as "degrees Brix" and is equivalent to percentage.

- Constant ºBrix level indicated that the fermentation process had stopped.



A.MALERA CULTURE



B.BREWER'S YEAST

FIG 13 Brix level of juice containing different yeast strains

- The graph showed that the malera product had less % total solouble solids left in the juice.

8. Standard curve for sugar estimation using DNS test.

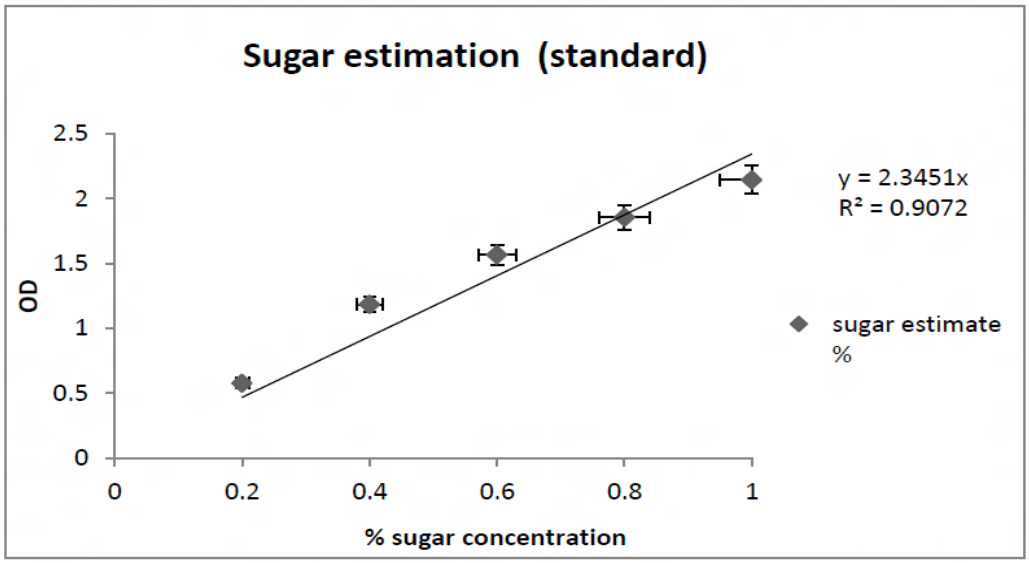
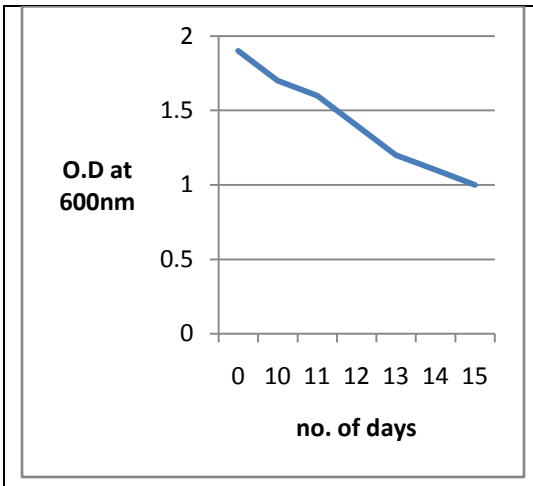


FIG 14 Standard plot of sugar concentration

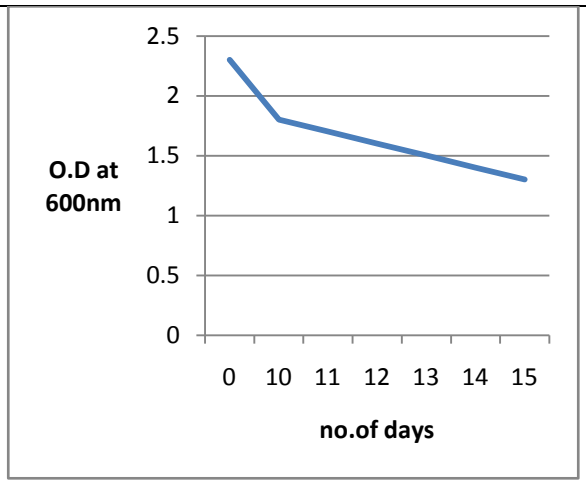
This standard curve was further used to estimate % sugar concentration in the fermented products.

9. Residual sugar estimation

Residual sugar is referred as the sugars that the yeast did not ferment or sugar that the winemaker added after the wine fermented, or both. If the amount of RS(residual sugars) is less then it has higher alcohol levels, is considered dry.



A.MALERA CULTURE



B.BREWER'S YEAST

FIG 15 Residual sugars in fermented juice at different time intervals.

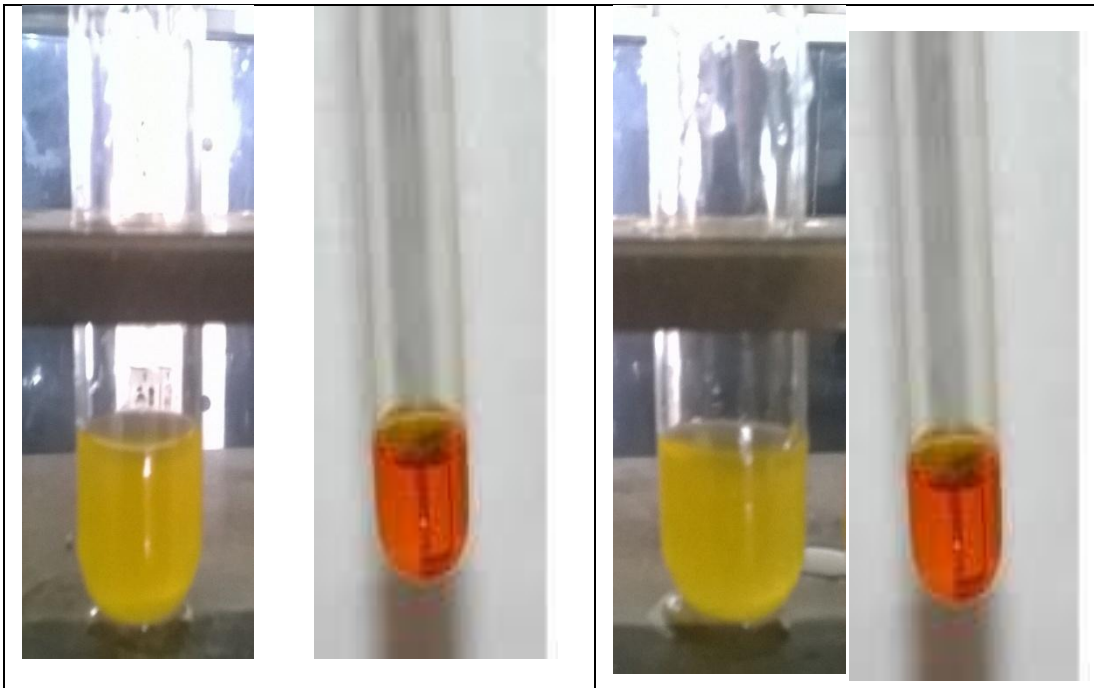
The graphs showed that for the malera culture, the residual sugars after the fermentation were less, i.e., the product was dry and had higher amount of higher alcohols.

- Constant residual sugar stated that the yeast could no longer ferment the juicet and the fermentation process had stopped.
- From the graph it can be inferred that the product with malera strain had the least % Residual sugar followed by the product with *Saccharomyces cerevisae* strains.
- This meant that the product with Malera had more higher alcohols and was more dry followed by the products with *Saccharomyces cerevisae* strains.

10. Qualitative estimation of ethanol in the juice.

On addition of potassium dichromate to the samples there was color change from orange to yellowish green which indicated the presence of ethanol in the samples. Potassium dichromate when reacted with ethanol converted to yellowish green color due to oxidation of dichromate into chromium ions.

Therefore, color change indicates the presence of ethanol in the sample.



A.MALERA CULTURE	B.BREWER'S YEAST

FIG 16 Qualitative test for ethanol production in juice fermentation

11.Quantitative estimation of ethanol

The alcoholometer was used to estimate the total ethanol content in the fermented litchi juice.

NAME OF CULTURE	%ETHANOL IN SAMPLE
Malera	30
Brewer's yeast	15

FIG 17 Alcohol content of different strains

Therefore, alcohol content was found to be two times higher in fermented juice with malera culture as compared to the wine prepared from commercial yeast culture.

CHAPTER 5
CONCLUSION

Malera culture shows better oenological properties than commercially available *Saccharomyces* yeast for the preparation of wine.

- Malera resulted in positive to flocculation test which is helpful for the easy recovery of final product thus decreasing the cost of the downstream processing and further decreasing the cost of final product.
- Malera starter has high ethanol tolerance capacity which means that it has better fermentation capacity and would allow the formation of more ethanol per batch.
- By using Malera culture, wine have less amount of residual sugars left behind which further enhances its fermentation ability and reduced cost of production.
- Wine prepared from malera culture have higher amount of alcohol content with the same amount of sugar used.
- There is a huge scope for product diversification for malera based drinks and food items. The starter could be used over other strains to get a product rich in alcohol content.
- Additionally, development of nutrabeverages from malera culture could form a good matrix for the therapeutic and nutritionally active constituents and would be a measure of sustainable NTFP management for tribal development.
- Using malera culture for the production of alcoholic beverages will reduce the cost of production.

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BRIEF BIODATA OF STUDENT

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