INTEGRATED ENOLOGY- CONVERSION OF WINERY BY-PRODUCTS INTO HIGH VALUE-ADDED PRODUCTS.

By-

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DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

JAYPEE UNIVERSITY OF INFORMATION TECHNOLOGY – WAKNAGHAT

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CERTIFICATE

This is to certify that the work entitled, "***Integrated Enology- Conversion Of Winery By-Products Into High Value-Added Products.***" submitted by- **Surabhi Soni (091710)** in partial fulfillment for the award of degree of Bachelor of Technology in Biotechnology of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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SUMMARY

For the past centuries, mahua flowers have been known for the production of distilled liquor by the tribal and rural people of India, which is hazardous to health. Application of fermentation process for making wine from mahua flowers is not common. This study was aimed to improvise and standardise the method of alcoholic fermentation and to develop a value-added product. Guava was chosen for value addition with mahua must. The alcohol content of the product was 8.0 - 9.0 % with higher alcohol content of 0.03, <0.01 and <0.01 % w/w for C3, C4 and C5 respectively. There is a huge scope for product diversification for mahua based drinks and food items. Additionally, development of nutrabeverages from mahua flowers could form a good matrix for the therapeutic and nutritionally active constituents. The report describes the standardisation of a value-added alcoholic fermentation of mahua flower must. Additionally, it also evaluates the effects of fermentation on physico-chemical parameters and biochemical characteristics.

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Dr. Gargi Dey

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LIST OF ABBREVIATIONS

ABBREVIATIONS	EXPANDED FORM
ml	Milli litres
mg	Milli grams
mins	Minuites
hr	Hour/ hours
rpm	Rotations Per Minuite
nm	Nano meter
%	Percent
w/v	Weight/volume
v/v	Volume/volume
TPC	Total Phenolic Content
µg	Micro grams
°C	Degree Celsius
NTFP	Non –Timber Forest P roducts
YPD	Yeast Peptone Dextrose
DNS	Di –nitro salicylic acid
YPDA	Yeast Peptone Dextrose Agar
RWPC	Red Wine Polyphenolic Compounds
gm	Grams
aq	Aqueous

CHAPTER 1

INTRODUCTION

Enology, a word that comes from the Greek words for wine and study, is the term used to describe the science of winemaking [1]. 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 [2] are :

a. *Carbon/ Energy sources:* This means the supply of soluble sugars in the juice. *Saccharomyces cerevisae* is capable of fermenting only a few simple sugars, and of those found in wine, only glucose and fructose are fermentable. High sugar content may cause difficulty, initially due to osmolarity of the juice and later, due to the combined effects of ethanol and the osmolarity of the must.
b. *Nitrogen content*: Some fruits like grapes have ample ammonium or amino nitrogen to complete fermentation. Excessive pre- fermentative clarification of the must can require the addition of ammonium salts which indirectly affects the aromatic character of wine.
c. *Lipids*: They play many important roles in yeasts, including nutrient storage and regulation. However, relative to fermentation, their main significance involves cell membrane function.
d. *Phenolic compounds*: In the production of wine, the type and quantity of phenolics play a major role in the quality of wine. Anthocyanins, flavonols, catechins and other flavonoids contribute to the sensory characteristics of wine, particularly colour and astringency; in addition, they possess a wide range of antioxidant and pharmacological effects. Phenolics vary notably according to several parameters such as the fruit variety, the maceration temperature, aging time. During ageing, phenolics evolve and monomeric anthocyanins polymerize by reaction with other flavonoid compounds and aldehydes [3-4].
e. *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. This value is particularly noticeable as the fermentation temperature rises. Fermentation usually ceases at between 13% - 15 % ethanol, while yeast growth generally stops at about half this value.
f. *Oxygen*: It is itself not required for fermentation. Nevertheless, oxygen uptake during crushing favors the production of essential sterols.

g. *Carbon dioxide*: A major by- product of fermentation. Except for sparkling wines, its retention is not desired. The escape of carbon dioxide is estimated to carry off the heat produced during fermentation. The gas also removes ethanol and aromatics from the wine.
h. *Temperature:* It can influence fermentation by affecting the rate of enzyme action.

Post fermentation processes:

Once the fermentation is complete, wine may undergo several processes which intend to assure that the wine remains clear and spoilage free after bottling, those used to adjust its colour, taste and flavor characteristics, and those designed to promote maturation and proper ageing.

Clarification- It refers to the procedures intended to assure that the wine remains crystal clear and spoilage free for bottling. For this centrifugation and filtration steps can be followed.

Ageing and Maturation-

Sur lies maturation is an old procedure enjoying renewed interest and application. It involves leaving the wine in contact with the lees for a period of three to six months.

Oak – barrel maturation adds an element of flavor complexity and adds a marked fragrance.

Health benefits of wine:

Wine although an alcoholic beverage is a healthy drink with a lot of therapeutic values. The favorable effect of moderate intake of alcohol results to its action on lipid profile, homeostatic parameters and reduction of inflammation markers.

Red wine has additional favorable effects presumably because of its high levels of polyphenolic compounds with antioxidant properties [5].

Red wine polyphenolic compounds (RWPC) exert numerous effects including free radical scavenging properties, anti-aggregatory platelet and anti-thrombotic activities. Moreover, RWPC are power vasodilators and contribute to the preservation of the integrity of the endothelium and inhibition of smooth muscle cell proliferation and migration.

Light to moderate intake of red wine produces a kaleidoscope of potentially beneficial effects that target all phases of the atherosclerotic process, from atherogenesis (early plaque development and growth) to vessel occlusion (flow-mediated dilatation, thrombosis). Such beneficial effects involve cellular signaling mechanisms, interactions at the genomic level, and biochemical modifications of cellular and plasma components. Red wine components, especially alcohol, resveratrol, and other polyphenolic compounds, may decrease oxidative stress, enhance cholesterol efflux from vessel

walls (mainly by increasing levels of high-density lipoprotein cholesterol), and inhibit lipoproteins oxidation, macrophage cholesterol accumulation, and foam-cell formation. These components may also increase nitric oxide bioavailability, thereby antagonizing the development of endothelial dysfunction, decrease blood viscosity, improve insulin sensitivity, counteract platelet hyperactivity, inhibit platelet adhesion to fibrinogen-coated surfaces, and decrease plasma levels of fibrinogen and coagulation factor VII [6].

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%.

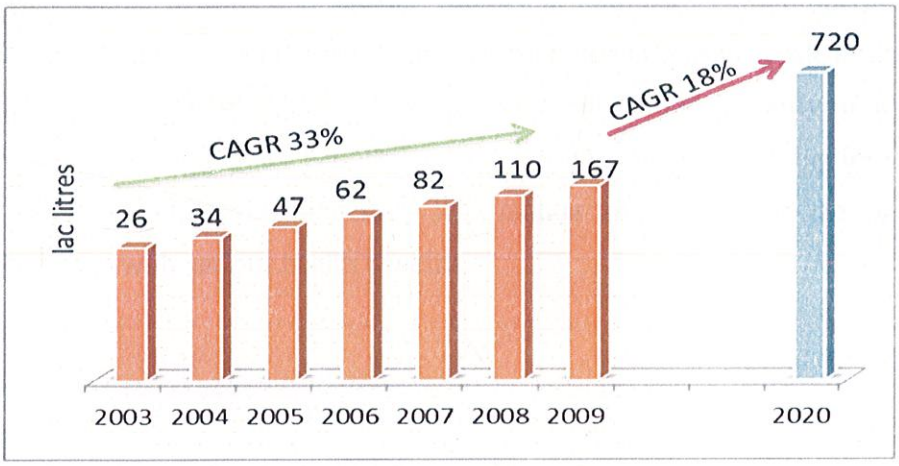


FIG 1.1 Compounded Annual Growth Rate Of Wine Industry In India Till 2020

- Growth of the domestic wine industry has provided significant employment opportunities to unskilled labourer.

(Source- Holkar and Jadhav, All India Wine Producers Association, Headquarters Worli, Maharashtra)

Need of hour

- Improved wine products with health benefits and better organoleptic qualities.
- Integrated processing approach for conversion of by- products into value- added products.

The new dietary habits and rising trend in production and consumption of designer foods have an environmental, health and social impact. In the present time, life style diseases such as obesity, cancer, diabetes, aging problems and degenerative diseases have been on the rise. Free radicals have been implicated in all these life style diseases. Antioxidants play a significant role in control of these diseases as they prevent or delay the oxidation of easily oxidizable substrates. This has led to an upsurge of recent interest in antioxidant rich natural beverages like tea, coffee and wine.

Today, nutrient fortified beverages are becoming a common feature but the high content of secondary metabolites present in fruits including phenolics, minerals and vitamins which give them both therapeutic and nutritional quality, have brought the focus back on fruits and vegetables. It is well known that red wine phenolics and flavonoids have a therapeutic potential. This has spurred the researchers to explore the potential use of other fruits with higher phenolic content compared to grapes, for the production of nutraceutical wines. The health promoting capacity of these fruit wines depends on various factors like the environment in which fruits are grown, time of maceration and fermentation, maturation, bottling and ageing. The colour and therapeutic properties is due to the presence of flavonoids and their derivatives and any technique that can result in the extraction of these compounds during wine making, will improve the nutraceutical quality of wines and make them a better product in the market [7]. Thus, standardised technology is required to explore the untouched potential of other raw materials like mahua and guava for the production of a nutraceutical beverage with various health benefits.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Mahua *(Madhuca latifolia)*

Commonly known as mahua is an Indian tropical tree found largely in the central and north Indian plains and forests. It is a fast-growing tree that grows to approximately 20 meters in height, possesses evergreen or semi-evergreen foliage, and belongs to the family Sapotaceae. It is adapted to arid environments, being a prominent tree in tropical mixed deciduous forests in India in the states of Chhattisgarh, Jharkhand, Uttar Pradesh, Bihar, Maharashtra, Madhya Pradesh, Kerala, Gujarat and Orissa.

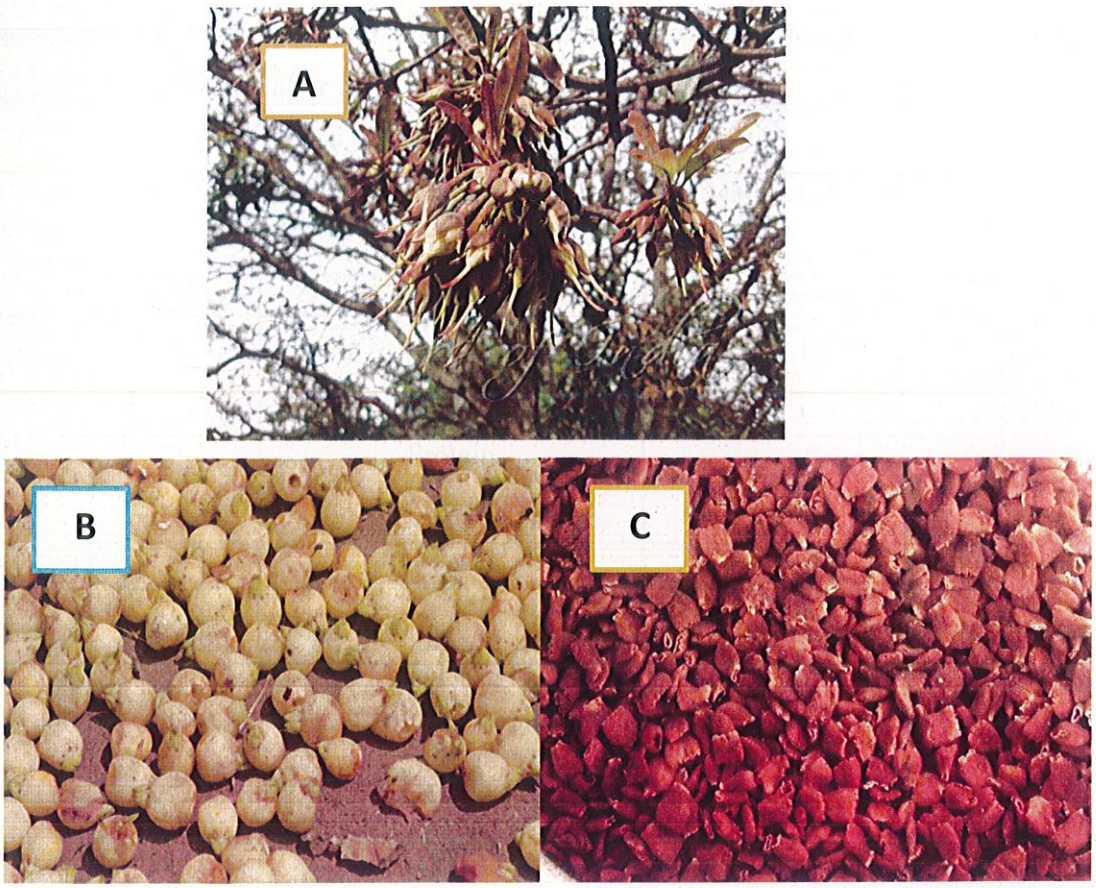


FIG 2.1 (A) Mahua flowers on tree, (B) Fresh mahua flowers and (C) Dried mahua flowers

Non-Timber Forest Products (NTFPs) are by far, one of the biggest resources which can be utilized for development of such nutraceutically enhanced products.[8-10]. Among the NTFPs, mahua (Madhuca indica) tree contributes significantly to the socio-economic status of the ethnic tribes of

India. Mahua flower contains total sugars (72.9 %) including arabinose, rhamnose, glucose, fructose and sucrose, carbohydrates (22.7 %), protein (4.4 %), starch (3.6 %), vitamin A (39 IU), fibre (1.7 %) and fat (0.5 %), calcium (0.14 mg/100 gm), and ascorbic acid (7.0 mg/100 gm) [11]. Apart from this, the Indian system of medicine (Ayurveda), has reported that the flowers have cooling, aphrodisiac, carminative and expectorant activities. They are also beneficial in indigestion and intestinal disorders [12].

Mahua flowers are a rich source of sugar, vitamins and calcium and offer wholesome nourishment when boiled with rice. Efforts are on to encourage the consumption of Mahua flowers in its processed form in urban areas. In view of their high sugar content and absence of any toxic effects, the flowers of Mahua are traditionally eaten by tribals and for other rural people. The mahua flower is used for curing many common ailments as well. The alcohol made from the flower can be used to cure bronchitis. If the flower is fried in ghee it helps cure piles. The flowers, which dry on the trees, can be used to treat malaria after it is made into a paste with water.

. **TABLE 2.1** Chemical Composition Of Mahua Flowers

S.No	Constituents	Percentage
1	Moisture	18.0
2	Protein	6.4
3	Fat	0.4
4	Total sugar	70.0
5	Fibre	1.7
6	Ash	2.7
7	Minerals, vitamins & others	0.8

In spite of nutritional and therapeutic benefits of the mahua flowers, it has not been sufficiently utilised. Mahua is hygroscopic and a large part of the collection is wasted due to lack of proper storage facilities. An estimated 90 % of annual production of mahua flower is fermented and distilled which gives spirituous liquor also known as 'country beer' [13]. The traditional processing practices produce distilled liquor which is frequently adulterated and of low quality and unfit for human consumption and health-hazardous causing incidents of mass poisoning resulting in death or irreversible eye damage [14].

There is a huge scope for product diversification for mahua based drinks and food items. Additionally, development of nutrabeverages from mahua flowers could form a good matrix for the therapeutic and nutritionally active constituents and would be a measure of sustainable NTFP management for tribal development.

2.2 Guava (*Psidium guajava*)

It is one of the most important fruits in India. It is one of the exotic fruits prized for its very pleasant, sub acidic and aromatic pulp. Guava, known as the poor man's apple of the tropics has a great potential for extensive commercial use because of its ease of culture, high nutritive value and popularity of processed Guava products.

Fermented guava fruit is also helpful in curing diabetes. Guava as a fruit is highly perishable, being susceptible to bacterial and fungal contamination, thus leading to their spoilage, mechanical damage and over ripenes. Hence, it is difficult to keep for long and are utilized either as fresh or processed into juice and speciality products.

It also exhibits antioxidant and free radical scavenging capacity [15]. Another benefit of guava is its therapeutic effect against prostate cancer and in reducing the cholesterol level due to presence of lycopene (potent antioxidant) and pectins respectively [16]. The fruit also contains high concentrations of Vitamin A (200-400 IU), ascorbic acid (88.2-250.8 mg/100 gm), total sugars (10-15.3 %), lycopene (45.3 µg/g Fresh Wt.), acids (10-15.3 %), phenols (170-345 GAE/g Fresh Wt.) and pectin (0.62 %) [17]. Moreover, the guava fruit has a major aromatic compound; Quercetin-3-O-alpha-l-arabinopyranoside (guaijaverin) which would mask the unpleasant flavour of mahua and improve the sensory qualities of the fermented product [18].

This necessitates the need for alternative preservation and post-harvest technologies towards their value addition that can reduce the level of post-harvest losses besides increasing diversity of wines.

2.3 Starter cultures

The strain used during fermentation can have a great influence on the ultimate quality of the end product, making the choice of yeast strain crucial if good quality fruit wines and distillates are to be assured.

Saccharomyces strains - During wine fermentation, yeast cells are subjected to a number of stresses, the most important being osmotic and ethanol stresses. Thus, yeast strains tolerant to high alcohol or glucose concentrations are used.

Non –*Saccharomyces* strains - These are normally used as mixed starter culture strains or in sequential fermentations as they contribute to the aroma properties and chemical composition of the resulting wine because more secondary metabolites are produced, which contribute to the taste and flavor of wines [19].

Malic acid, which is the major organic acid in fruit musts is sometimes detrimental to the quality of wines when present at high concentrations. For this purpose, *Issatchenkia orientalis* was used as it could degrade malic acid rapidly.

2.4 Organoleptic or sensorial properties

- Ethanol tolerance
- Osmotolerance
- Invertase activity
- Amylase activity
- Releasing of cell wall polysaccharides and manoproteins
- High fermentative power
- Low production of volatile acidity (acetic acid)
- Low production of toxic compounds (methanol)
- Degradation of malic acid
- Others include- taste, appearance, color, aroma, body and firmness.

2.5 By-products of winemaking process

There are two processes which give by-products during winemaking and these are-

- By products formed during fruit or substrate processing

  It mainly consists of fruit peel, seeds, fibers, etc. these are important as they are rich source of anti-oxidants such as anthocyanin pigments, tannins, catechins…etc

  Yellow fruit peels have xanthin, carotene and luteine. Some have pectins, tannins, gum , etc. These compounds increase bulk of the food and helps prevent constipation by reducing gastro-intestinal transit time. They also bind to toxins in the food which helps to protect the mucus membrane of gut and thus cuts colon cancer risk. Furthermore, dietary fibers bind to bile salts (produced from cholesterol) and decrease their re-absorption, thus help lower serum LDL cholesterol levels.

  Guava skin contains contain both carotenoids and polyphenols like gallocatechin, leucocyanidin and amritoside –the major classes of antioxidant pigments – giving them relatively high potential antioxidant value among plant foods.

- By-products of fermentation process

  Yeast lees is the major by- product.

  Lees refers to deposits of dead yeast or residual yeast and other particles that precipitate to the bottom of wine after fermenting and aging.

  Autolysis in winemaking relates to the complex chemical reactions that take place when a wine spends time in contact with the lees, or dead yeast cells, after fermentation. The effects of autolysis on wine contributes to a creamy mouthfeel that may make a wine seem to have a fuller body. The release of enzymes inhibits oxidation which improves some of the aging potential of the wine.

  Spent yeast is particularly high in substances called polyphenols, found in the cell walls of the yeast. These can be used in dietary supplements. Manufacturers use either hot water and organic solvents, or an enzyme treatment to extract the polyphenols from the wine lees. When added to a supplement, these substances are said to stimulate immune response against infections and even cancer.

2.6 Volatile compound analysis

A number of methods in the form of hedonic scales and analytical techniques like GCMS have been developed The volatile compounds are higher alcohols (fuel alcohols) and esters which increase the wine flavor complexity.

2.7 Wine and polyphenols

Phenolic components greatly contribute to the organoleptic characteristics of fermented products like wine such as color, astringency and aroma [20]. Currently, the berry phenolics are also associated with many beneficial physiological effects like protective effects against oxidative stress and hypercholesterolaemia [21].

Phytoestrogens could have a protective effect on the initiation or progression of breast cancer by inhibiting the local production oeoestrogens from circulating precursors in breast tissue. Amongst the phytoestrogens, the flavones and flavonones are the most potent inhibitors of aromatase. Red wine polyphenols inhibit aromatase activity[22].

The polyphenols in wine help in limiting the initiation and progression of atherosclerosis and regular but moderate alcohol consumption levels of high-density lipoprotein (HDL) cholesterol, or the "good cholesterol." remove low-density lipoprotein (LDL) cholesterol, from the circulation and lessen the amount of material available for fatty plaque formation. [23].

Physiologically active plasma concentration of phenolic compounds is dependent on the bioavailability of polyphenols in humans. only 5% of the dietary polyphenol is absorbed in the duodenum. Over 95% of the intake passes to the colon and is fermented by the gut microflora. A fraction of the microbial metabolites is absorbed and appears in the plasma as mammalian conjugates.[24-27].

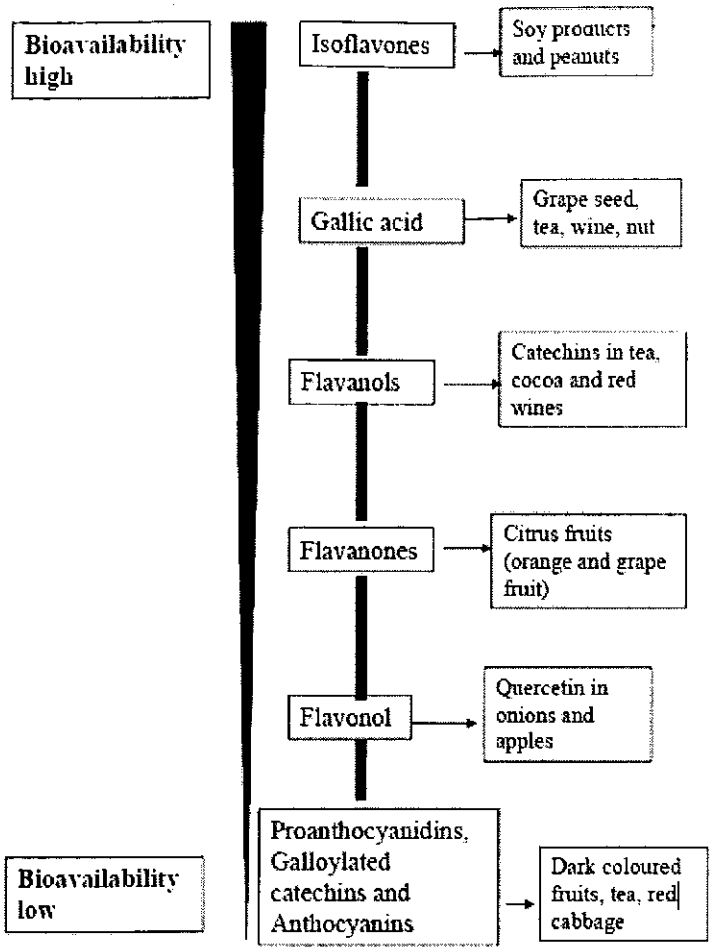


FIG 2.2 Bioavailability ranking of polyphenols [28].

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

1. ***Mahua flowers***- The mahua flower buds were collected from Rewa, Madhya Pradesh, India in the month of April-May and were air-dried. The samples were further sorted, cleaned and stored.

2. ***Guava fruit***- The ripened guava fruits were procured from the local market of Solan, Himachal Pradesh, India from the same vendor and were also sorted, cleaned and stored for further use.

3. ***Yeast strains***- The strains currently worked on in this project are-
   - The strain *Saccharomyces cerevisae* was obtained from a local brewery – Minchy's Food Products, Shoghi, Himachal Pradesh, India.
   - Lalvin EC-1118 (*Saccharomyces bayanus*)
   - Lalvin ICV-D47

4. ***Chemicals*** –

   a) Composition of media for maintenance and preservation of yeast cultures :
   - Yeast extract powder (HIMEDIA)
   - Peptone, bacteriological (HIMEDIA)
   - Dextrose anhydrous powder ($C_6H_{12}O_6$) (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 orthophosphate) extrapure AR (SISCO RESEARCH LABORATORIES PVT. LTD)

d) Pectinase from Aspergillus niger (P4716- 5KU) SIGMA.

e) Glycerol (CH2OH.CHOH.CH2OH) (Fisher Scientific)

f) Methanol (MERCK)

g) Ethanol (MERCK)

h) Chemicals required for DNS test :

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

i) Chemicals required for calculating total phenolic content of wine :

- Sodium carbonate (MERCK)
- Gallic acid (MERCK)
- Folin co- calteau's reagent (MERCK)

j) Chemicals required to check total acidity of wine :

- Phenolpthalin (MERCK)
- Sodium hydroxide pellets purified (MERCK)

3.2 METHODS :

1. *Maintenance and preservation of yeast culture*

(Yeast Peptone Dextrose Agar) YPDA 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), Agar- Agar (2.0gm) and Distilled water (100ml).

Slants were prepared from the autoclaved media and streaking was done on the slants using the source as the slant or overnight grown active dry culture obtained from the source. Once the slants were prepared, they were stored at 4°C until further use.

After every month, the slants were sub-cultured in order to prevent cell death due to nutrition depletion.

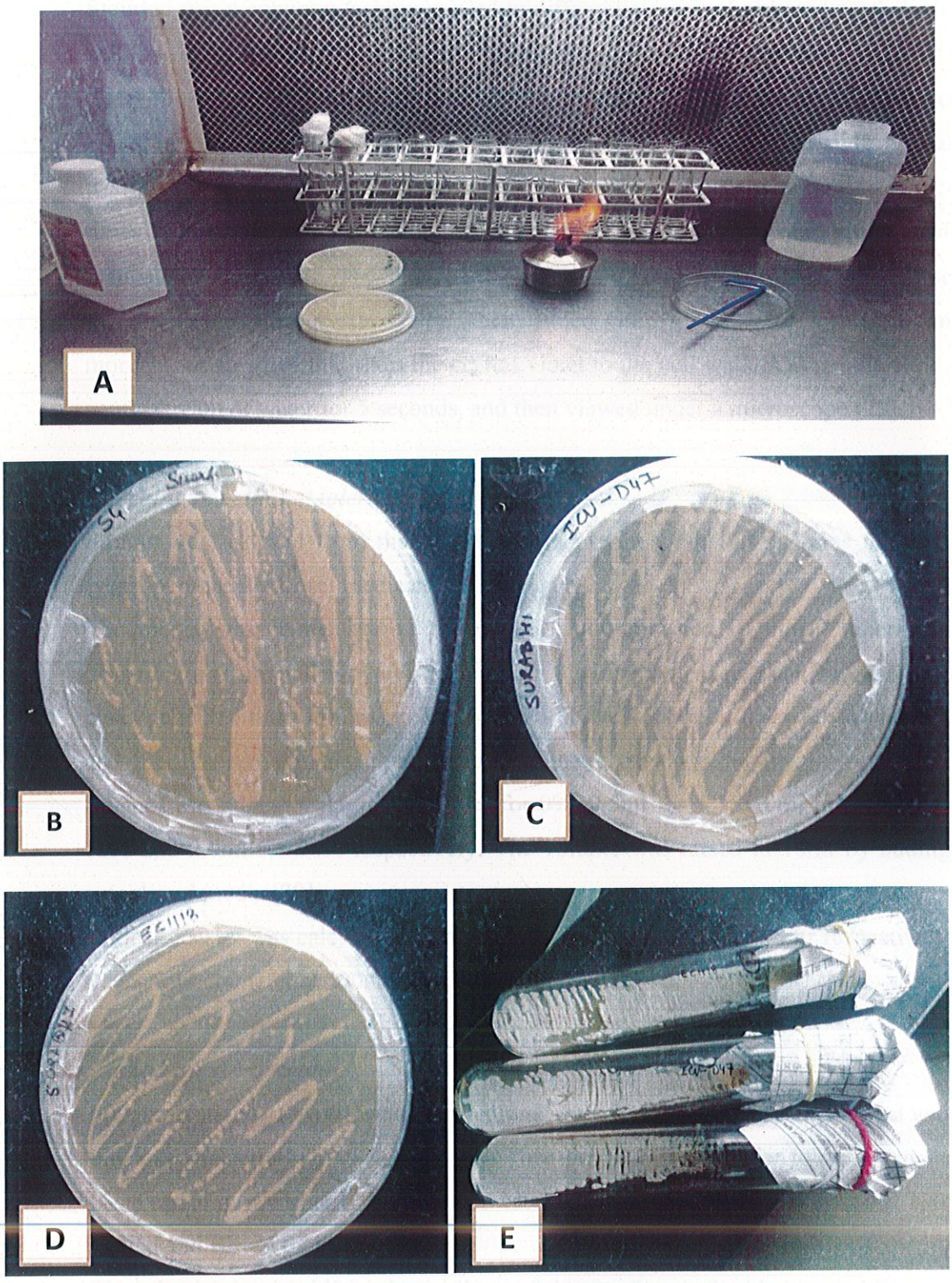


FIG 3.1 (A) Culturing of plates and slants in LAF, (B) *Saccharomyces cerevisae* plate, (C) Lalvin ICV-D47 strain Plate, (D) Lalvin EC1118 strain plate and (E) slants of strains

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. Slides 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 [29].

3. ***Checking the ethanol tolerance of strains***

Strains were revived from their slants and a pre-inoculum (24 hr) was made in YPD (yeast peptone dextrose) media at 37°C.

The media was centrifuged at the rate of 5000 rpm for 10 mins. Supernatant was discarded and the pellets were washed with distilled water and centrifuged again to get clean pellets. 5 test tubes marked as 0%, 5%, 10%, 15% and 20% were set for 0hr, 24hr and 48hr respectively. Inoculum added to all the tubes was 0.2% w/v.

Ethanol was added in the tubes as 0ml for 0%, 0.5ml for 5%, 1.0ml for 10%, 1.5ml for 15% and 2.0ml for 20% respectively. The volume was raised to 10ml by adding fresh autoclaved media [30].

Optical density was calculated for all the tubes at 0hr, 24 hr and 48 hr respectively using a spectrophotometer at 600nm. Work was done in triplets.

4. ***Mahua must extraction***

500gm of mahua flowers were washed and dried in the sun to get rid of dust and other unwanted material. Dried flowers were then grinded in a blender to get a powder or a paste out of it. It was then diluted with water in 1:2 (% w/v) ratio and boiled at 100°C.

Boiled and stirred for about 30 mins.

Then the extract was allowed to sediment for some time and with repeated filtration was done with the help of autoclaved muslin cloth.

The extract was centrifuged at the rate of 5000rpm for 10 minutes to clear the juice.

The extract/ must was then pasteurized at 90°C for 10 minutes and rapidly cooled down to room temperature by keeping it on ice.

The must was then stored at 4°C for future use in proper sealed bottles.

5. ***Guava must extraction***

500g of fresh guavas fruits were mashed manually for the preparation of must. The fruit pulp was diluted with water in 1:2 (% v/v) ratio and treated thermally at 90°C for 10 min.

6. ***Blending of mahua and guava must***

The mahua extract must was blended with diluted guava juice with a blender in the 1:1 (% v/v) ratio and was stored at 4°C for further analysis.

7. ***Pectinase treatement***

Mahua and mahua-guava must were both treated separately with pectinase at the rate of 1gm / 100ml for 24 hours. They were then centrifuged and given a thermal treatment at 90°C for 10 minutes in order to eliminate contamination. The pectinase-clarified mahua and mahua-guava blended musts were used individually for setting up of the fermentation.

8. ***Fermentation setup for mahua and mahua-guava must***

The alcoholic fermentation was setup by using mahua flower must and mahua-guava must 1:1 ratio (% v/v) separately. For the fermentation, 1 liter of mahua and mahua-guava must were used. Preliminary studies were performed to standardize the inoculum size and ratio of the starter culture. 0.2 %, w/v yeast was used to inoculate the must for each. Ammonium di-hydrogen orthophosphate (0.2 %, w/v) was added as the nitrogen source for the yeast. The fermentation was set up at 25°C. The fermentation process was monitored by the daily measurement of °Brix level of the product and it was terminated when the product reached a constant °Brix. Then put the setup at 3-4°C to stop the fermentation process and proceed for ageing or maturation of wine.

9. ***Downstream processing of mahua and mahua-guava blended fermented product***

To stop the fermentation process, the flask was kept at 3-4 °C undisturbed for 24-48 hrs and was allowed to undergo sedimentation.

After 24 hours, the fermented product was filtered using a funnel and 5-6 layers of autoclaved muslin cloth and again left for sedimentation for the next 24 hours.

Then the product was centrifuged at the rate of 5000rpm for 15 mins to get a clear liquid.

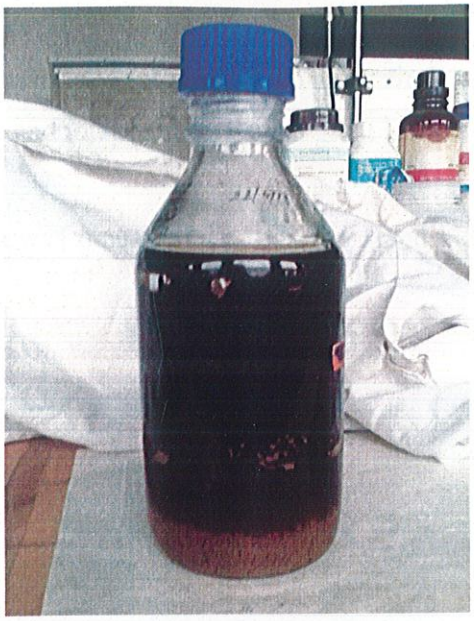


FIG 3.2 Downstream processing- fermented product

Pasteurization was done by putting the product in a boiling water bath (100°C) for 15 mins and rapidly giving it a cold treatment by putting it on ice to bring it to room temperature

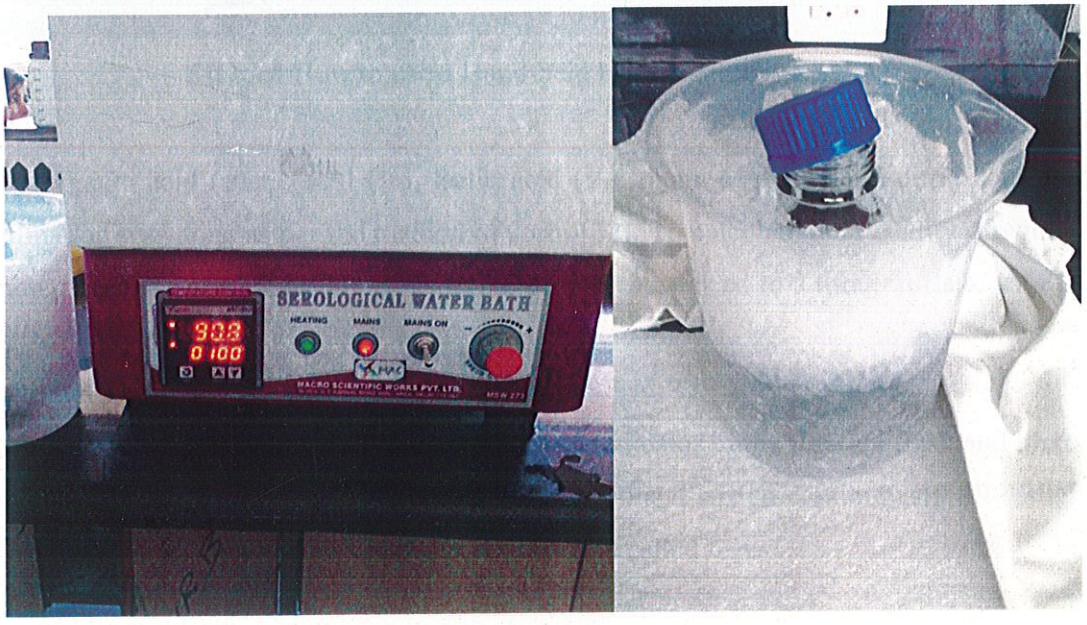


FIG 3.3 Downstream processing- pasteurization process

10. Physico-chemical analysis

The physicochemical characteristics of wines such as alcohol content, pH value, titratable and volatile acidity, color, and total extract, have a great influence on their overall quality [31].

Total soluble sugar (TSS) was measured as °Brix by Hand-held refractometer (ERMA, Japan).

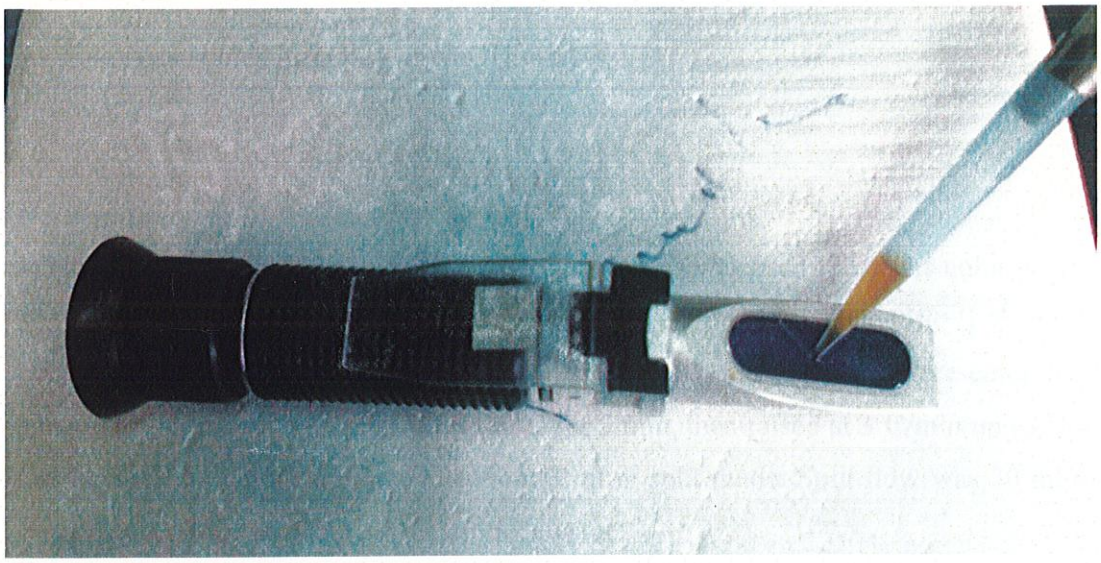


FIG 3.4 How to use a Hand-held Refractometer (ERMA)

The organic acid (acetic acid (%), lactic acid (%), malic acid (%) and citric acid (%)) estimation was done as per the method of Joshi [32]. To calculate total acidity of wine- a known quantity of sample (20ml) was filtered and transferred to a titration flask with the help of a pipette.

Take N/10 NaOH was taken in a 50ml burett

A few drops of phenolphthalein was added as an indicator in the titration flask and mixed well. N/10 NaOH was slowly released from burette and stirred continuously till a slight pink colour appeared. The volume of NaOH used is called titre.

Formula used-

$$Acidity\ \% = \frac{titre * N\ of\ NaOH * miliequivalent\ weight * 100}{Volume\ of\ sample} * dilution\ factor$$

Miliequivalent weight of acids-

Acetic acid- 0.0060

Lactic acid- 0.0090

Malic acid- 0.0067

Citric acid- 0.0064

Also here Normality of NaOH used is 0.1

11. Determination of higher alcohols by GC

A Hewlett-Packard 5890 Series II gas chromatograph was used [33]. The column (50 m, 30.32 mm and 0.5 mm film thickness) was a DB-20 from J&W Scientific (Folsom, CA, USA). The column was preceded by a 2m, 30.53 mm uncoated pre-column The temperature program was as follows: 40°C for 5 min, then raised at 3°C/min up to 200°C. Carrier gas was H at 3 ml/min. Injection: 3 ml in split mode. Split flow was 30 ml/min. analysis was made by Flame Ionization Detector.

12. Determination of Total Phenolic Content

a. The Folin-Ciocalteu method was used for the determination of total phenolic content against a gallic acid standard [34] with the use of the following chemicals – sodium carbonate, gallic acid and F-C reagent.

200gm of sodium carbonate was dissolved in 1litre of distilled water for stock solution.

1gm of dry gallic acid was dissolved in water and the volume was raised to 1000ml.

250µl of different concentrations of gallic acid was added to 15ml distilled water. Then 1.25ml of F-C reagent and 3.75 ml of sodium carbonate were added in each test tube. For the samples also instead of gallic acid add 250µl of wine sample.

Final volume is raised to 25ml by adding distilled water and vortexed well.

Incubated at 23.9°C for 2 hours and its optical density was recorded at 765 nm with the help of a spectrophotometer.

The OD measured at 765nm was plotted with their respective concentrations to get a standard plot

b. By the method of liquid – liquid extraction using an organic solvent

This method is used to check the phenolic content of wine.

Wine sample was mixed with an organic solvent in the ratio of 1:1 and placed at room temperature under darkness for 48 hr in a separation funnel.

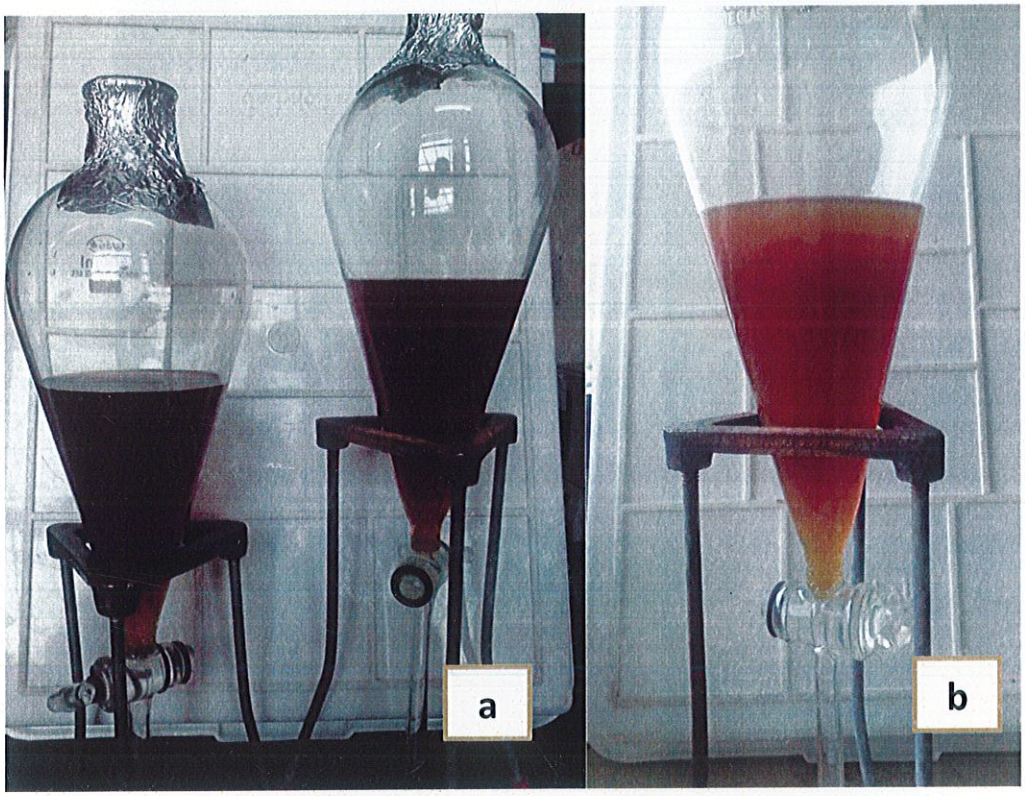


FIG 3.5 **a)**.Wine + organic solvent(methanol) in the ratio 1:1 in a separation funnel and **b)** liquid liquid extraction after 24 hours.

After 48 hr there was a complete line of separation between the two liquid phases. Then the organic phase was removed, placed in a round bottom flask and evaporated to dryness using a rotavac until all the solvent was dried with just the residue left in the flask.

The residue was recovered by 2-4 ml of water which was used for lab analysis and 2-4 ml of organic solvent which was extracted and sent for GC-MS analysis [35].

FIG 3.6 evaporation of the organic solvent using rotavac

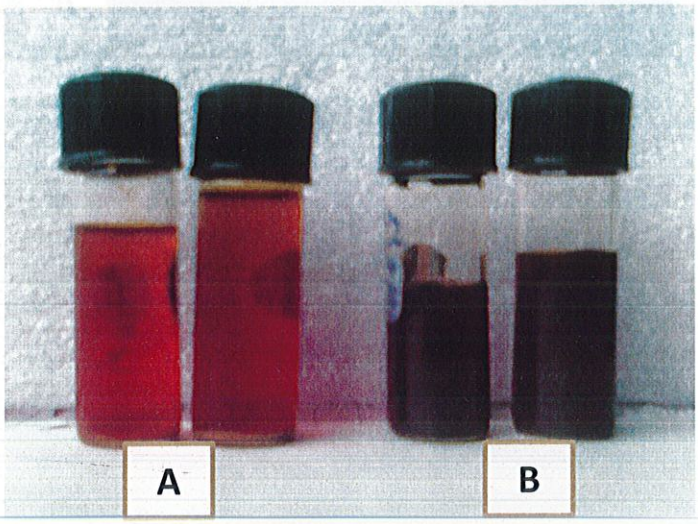


FIG 3.7 samples for calculating TPC by GC-MS. (A) in organic solvent (methanol) and (B) in aqueous solution.

c. By the method of solid- liquid extraction using an organic solvent

This method is used to check the phenolic content of dried biomass or dried lees.

The biomass from the wine was collected by regular filtration and centrifugation and allowed to dry on glass plates in an incubator. Everyday their weight was monitored till it became constant. It was then scraped out from the plate and kept away from moisture in sealed tubes.

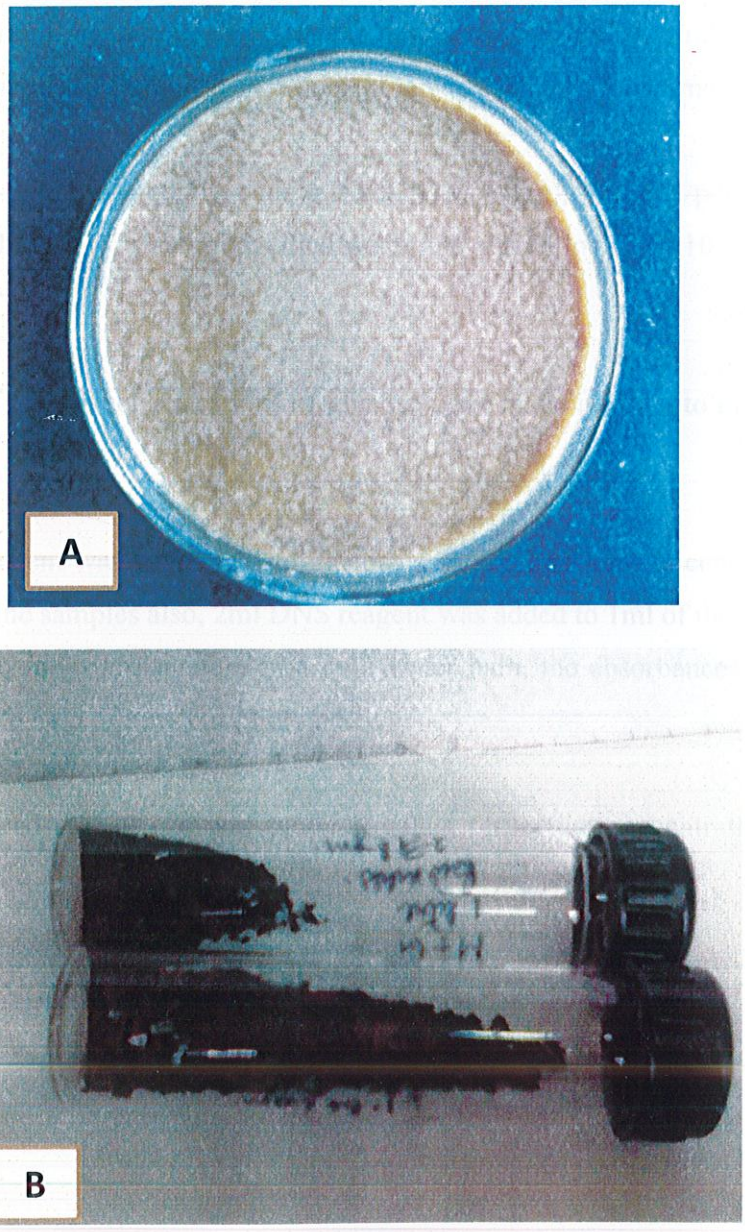


FIG 3.8 (A) Fresh biomass **(B)** Powdered and dry biomass for the extraction of polyphenols.

4gm of dried sample was mixed with 100ml of organic solvent with agitation at room temperature under darkness for 48 hr. Then the organic phase was removed, evaporated to dryness at less than 35°C using a rotavac and the residue was recovered by 2-4 ml of water for lab analysis or 2-4 ml of organic solvent for GC-MS analysis [35].

13. Sugar estimation using DNS test

The test by Miller [36] 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.

5ml solution of known concentrations of glucose were 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

4.1 Staining of different Saccharomyces strains

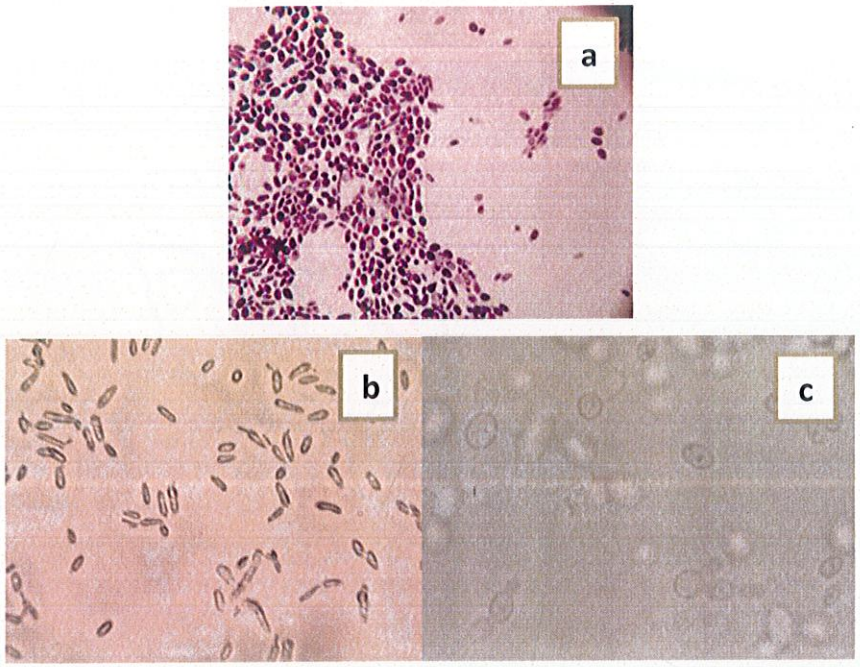


FIG 4.1 Stained slides of **a**. *Saccharomyces cerevisae.* **b**. *Saccharomyces bayanus* (EC-1118) and **c**. ICV-D47 strain.

4.2 SEM images of strains

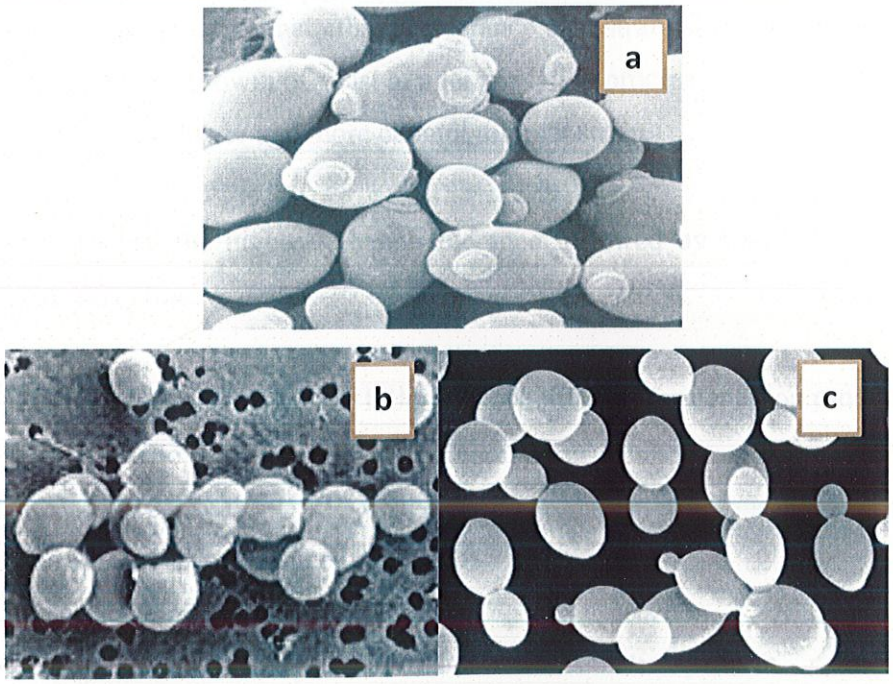


FIG 4.2 SEM images of a. *Saccharomyces cerevisae,* b. *Saccharomyces bayanus* (EC-1118) and c. ICV-D47 strain.

4.3 Ethanol tolerance of different strains

By checking the % survivability of all the strains in a nutrient media containing 0%, 5%, 10%, 15% and 20% ethanol at 48 hr , it was recorded that-

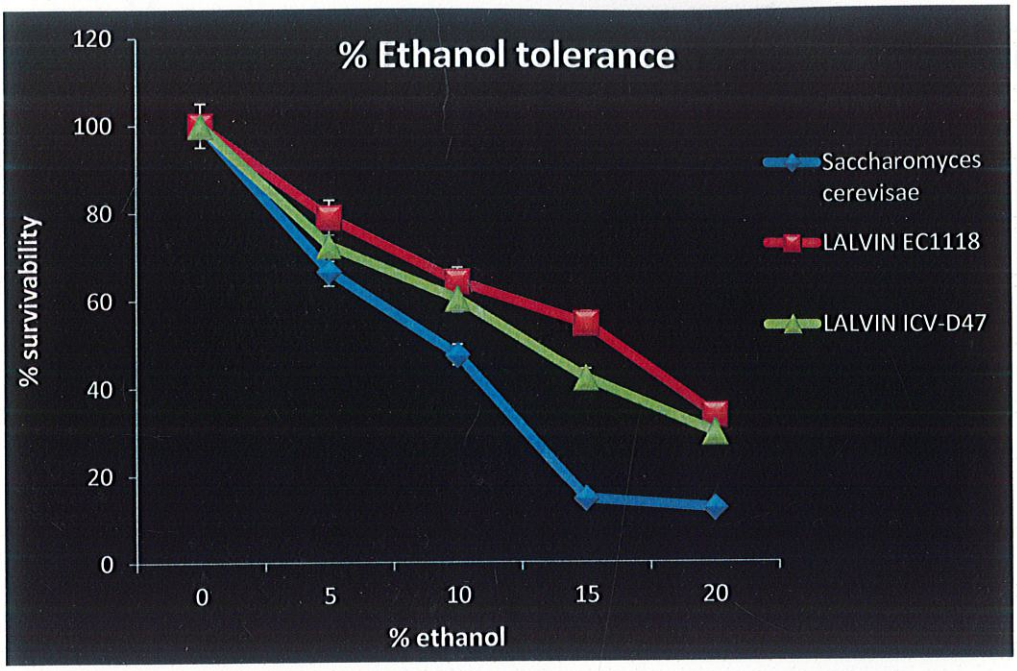


FIG 4.3 % Ethanol Tolerance of strains used

It can be inferred from the table 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.

- LALVIN EC1118 had the highest ethanol tolerance followed by LALVIN ICV-D47 and *Saccharomyces cerevisae.*

LAVIN EC1118 could be used to get the following beneitfs in a fermentation process-

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

4.4 Standardised curve for determining total phenolic content using Folin Co-Calteau's method.

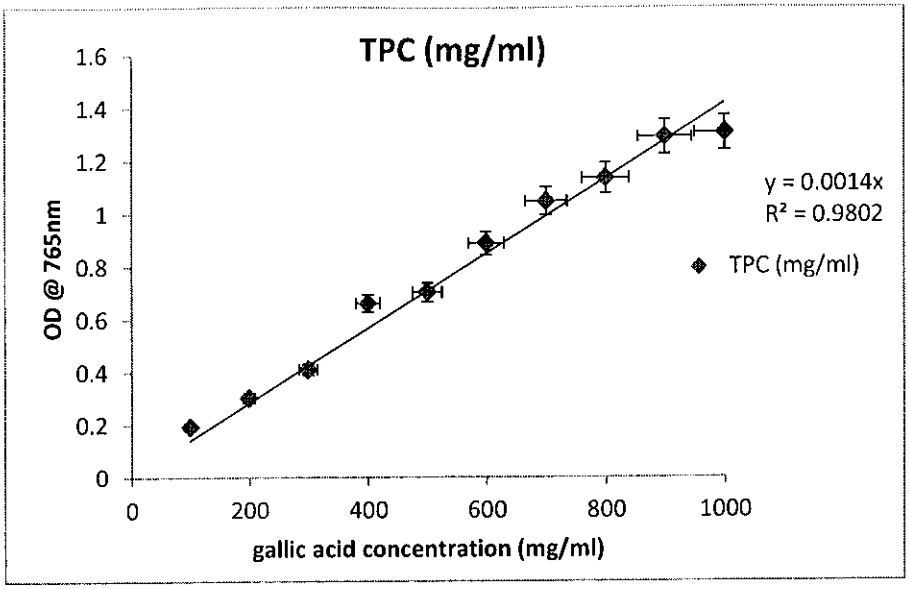


FIG 4.4 Standard plot for determining total phenolic content

This standard curve was further used to calculate TPC (mg/ml) of all the fermented products .

4.5 Standardised curve for sugar estimation using DNS test.

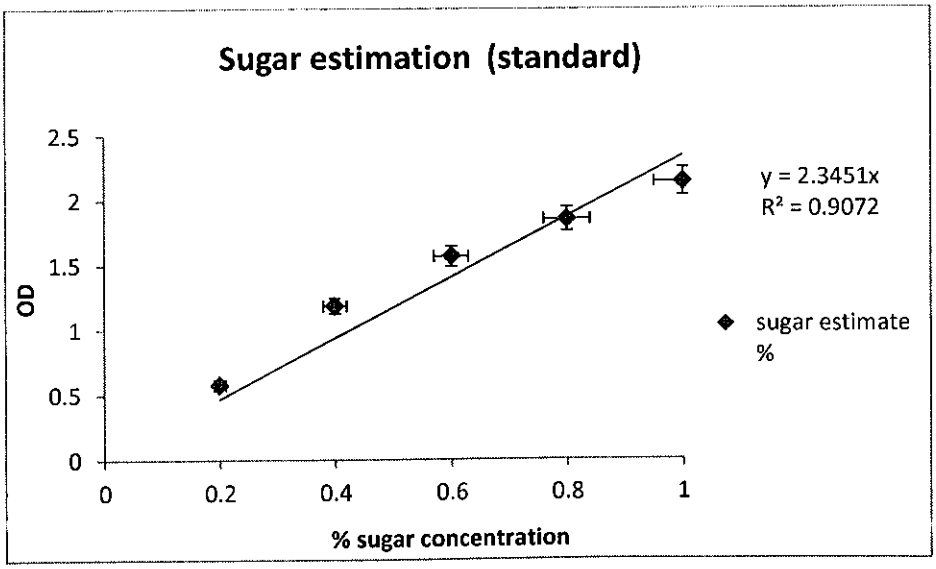


FIG 4.5 Standard plot for sugar estimation by DNS test

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

4.6 Standardisation Of Fermentation And Downstream Processing.

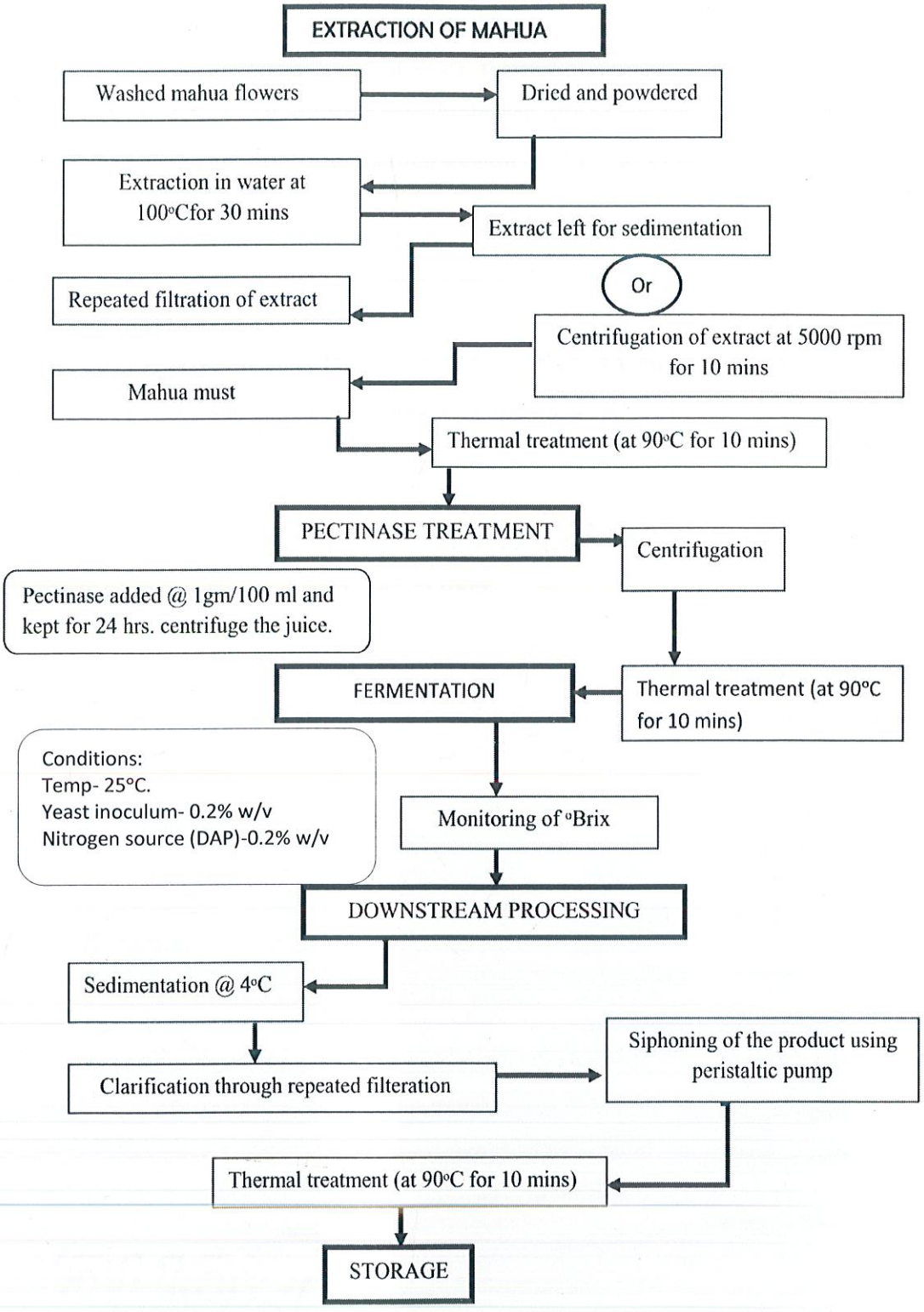


FIG 4.6 Standardised fermentation and downstream process.

The main aim was to improve the traditional brewing process to produce a fermented product with low alcohol content, enriched with nutraceutical components and with a potential in the market.

The pectinase treatment for clarification of must, thermal treatment of must for removal of pathogens, supplementation with diammonium hydrogen orthophosphate (DAP), use of a pure culture (*Saccharomyces cerevisae*) and thermal treatment of the fermented product to enhance the shelf life were the improvisations. The fermentation process was set up at 25°C.

Pectinase was added to mahua must for the clarification of must so that the viscosity of the juice was reduced and colloids were removed.
Clarification enabled more efficient fermentation, resulting in a wine with cleaner and fruitier aromas, removed tannins and reduced the danger of hydrogen sulfide (H_2S) formation.

The fermentation of mahua must (1L) and mahua-guava blend (4L) were setup with *Saccharomyces cerevisae* strain at 25°C for 15 days.

The fermentation of mahua-guava blend (1L) was setup with all the three strains, i.e., Saccharomyces cerevisae, LALVIN EC1118 and LALVIN ICV-D47 at 25°C for 15 days.

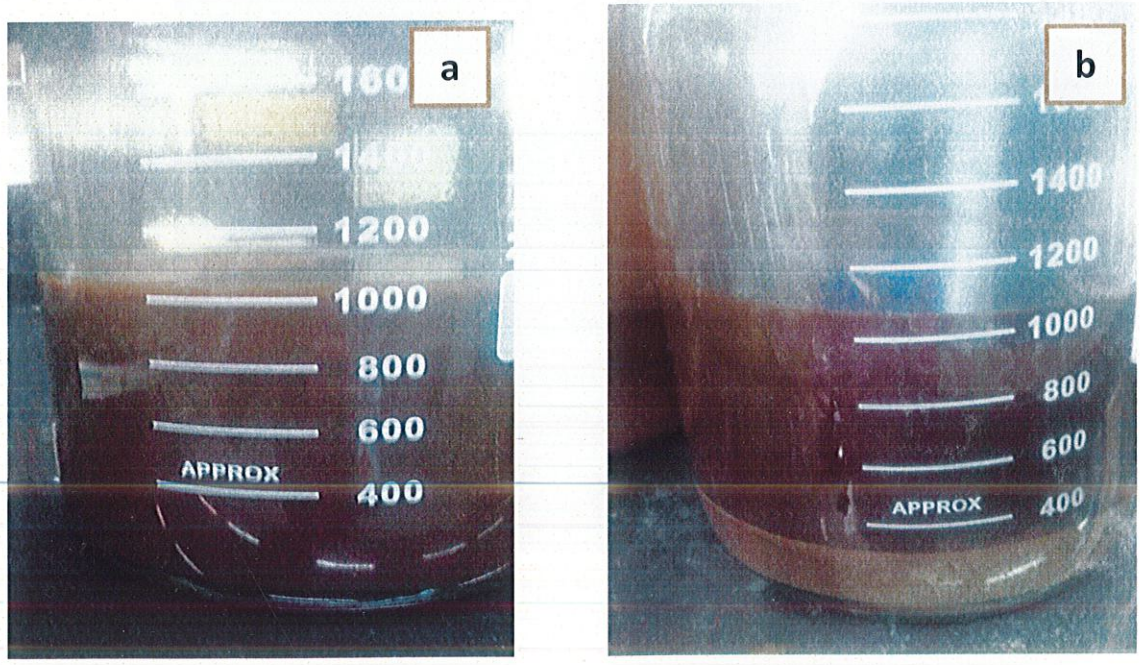


FIG 4.7 a. Mahua must and **b.** Mahua must after addition of pectinase

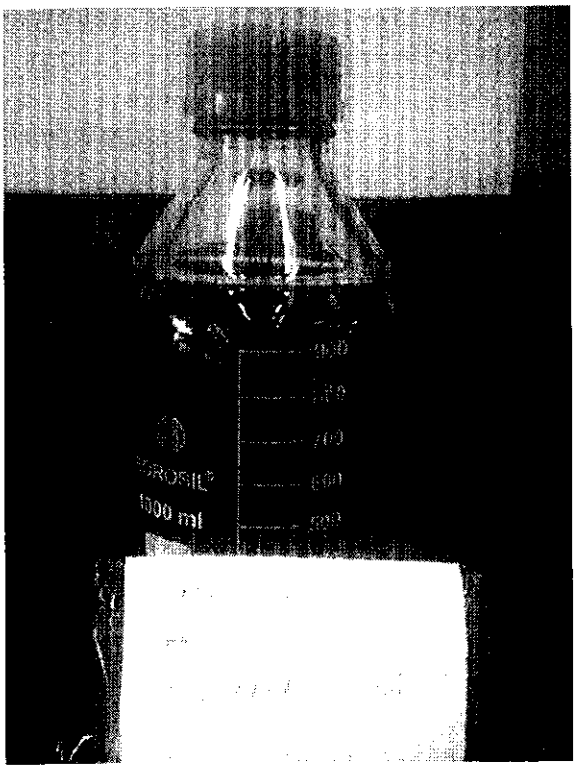


FIG 4.8 Mahua fermented product

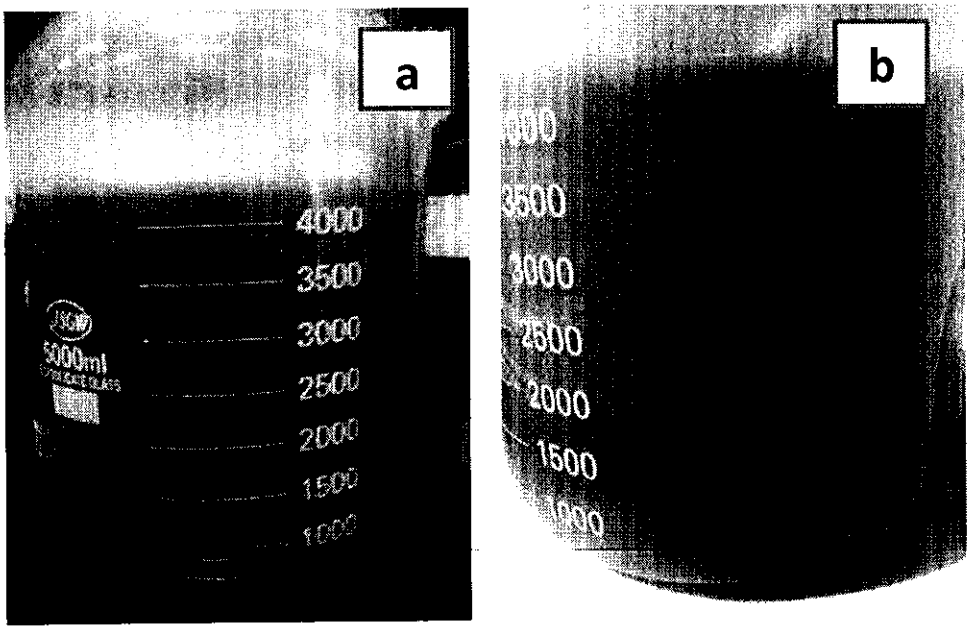


FIG 4.9 **a.** Mahua- guava must and **b.** Mahu-guava must after addition of pectinase

(A)

(B)

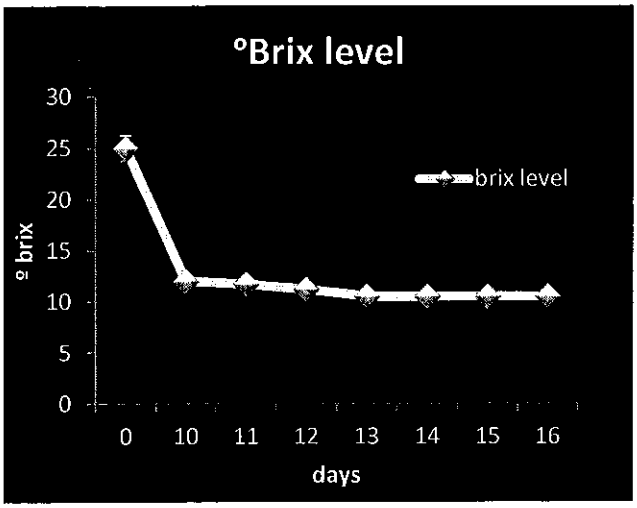
(C)

FIG 4.10 Mahua-guava (1L) fermented products with strains **(A)** *Saccharomyces cerevisae* **(B)** LALVIN EC1118 **(C)** LALVIN ICV-D47.

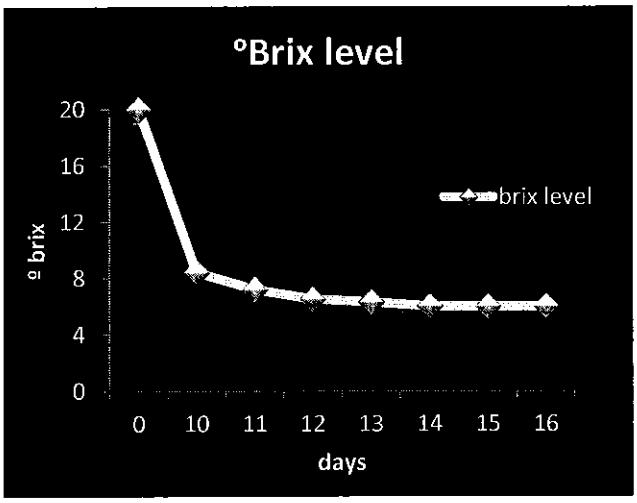
4.7 physico-chemical characteristics of fermented product

a. °Brix level estimation

This was used as a measure of the total soluble solids in the must and was measured with the help of a hand-held refractometer.



(A)



(B)

FIG 4.11 Monitored analysis for 15 days for °Brix level estimation
(A) Mahua (1L) setup **(B)** Mahua-guava blend (4L) setup

- 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 °Brix level indicated that the fermentation process had stopped.
- The graph showed that the mahua product had more % total solouble solids left in the must after the fermentation as compared to the mahua-guava blend.
- Blending or value addition decreased the amount of total solouble solids in the fermented product .

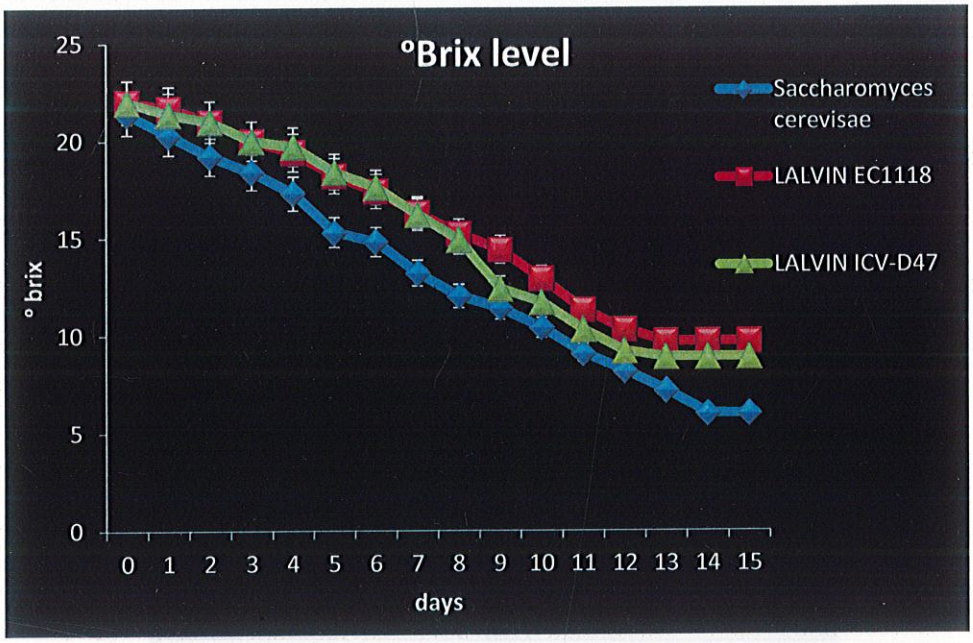


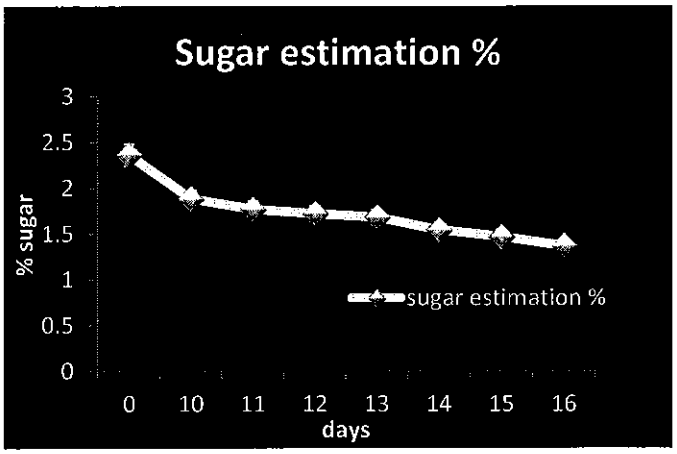
FIG 4.12 Monitored analysis for 15 days for °Brix level estimation of mahua-guava blends (1L) with 3 different strains.

- Constant °Brix level indicated that the fermentation process had stopped.
- The percentage sugar, measured in degrees Brix, indicated the sweetness of the wine by measuring the number of soluble solids in the must.
- The decrease in °Brix was highest as seen in fermented product with *Saccharomyces cerevisae* strain, this showed that the the total solouble sugars were the least this product followed by LALVIN ICV-D47 strain and LALVIN EC1118 strain.
- The amount of total solouble solids left showed that product with LALVIN EC1118 was the sweetest followed by the product with LALVIN ICV-D47 and *Saccharomyces cerevisae* strain.

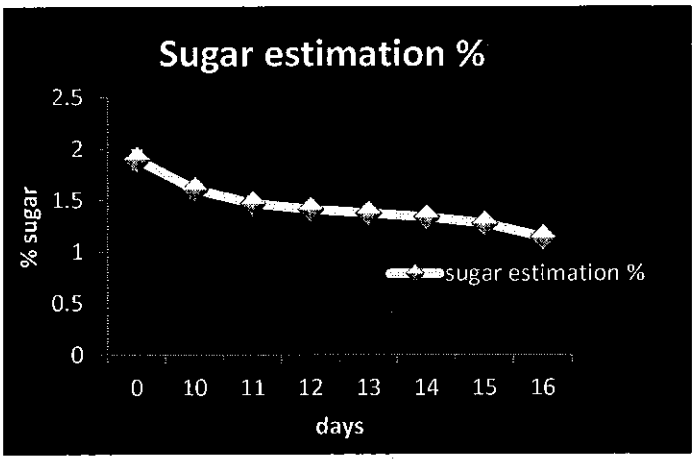
b. % Residual sugar estimation

Residual sugar is reffered 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, and is considered dry.



(A)



(B)

FIG 4.13 Monitored analysis for 15 days for % residual sugar estimation

(A) Mahua (1L) setup **(B)** Mahua-guava blend (4L) setup

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

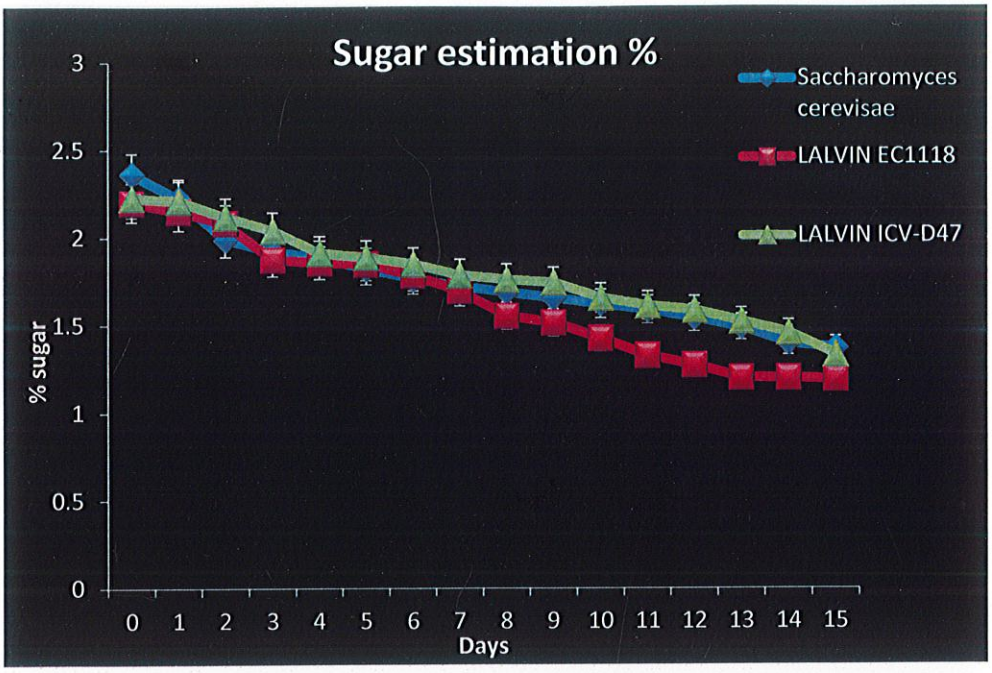


FIG 4.14 Monitored analysis for 15 days for % residual sugar of mahua-guava blends (1L) with 3 different strains.

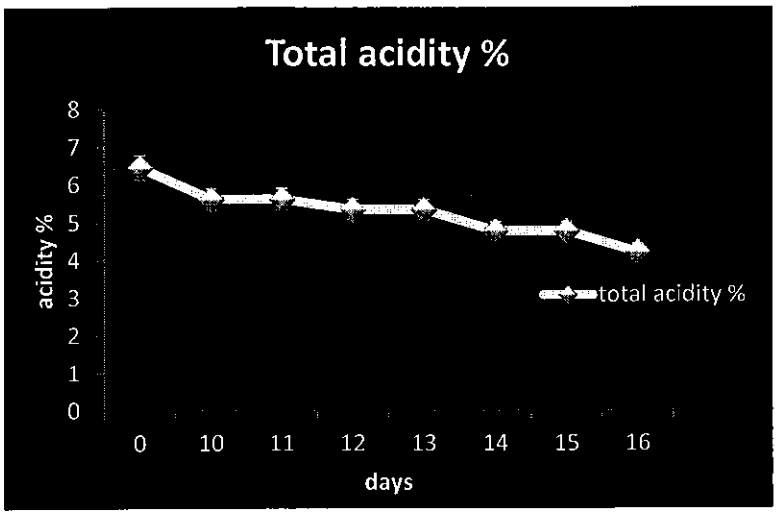
- Constant residual sugar % stated that the yeast could no longer ferment the must and the fermentation process had stopped.
- From the graph it can be inferred that the product with LALVIN ICV-D47 strain had the least % Residual sugar followed byt he product with LALVIN EC1118 and *Saccharomyces cerevisae* strains.
- This meant that the product with LALVIN EC1118 had more higher alcohols and was more dry followed by the products with with LALVIN EC1118 and *Saccharomyces cerevisae* strains.

c. % Total acidity estimation

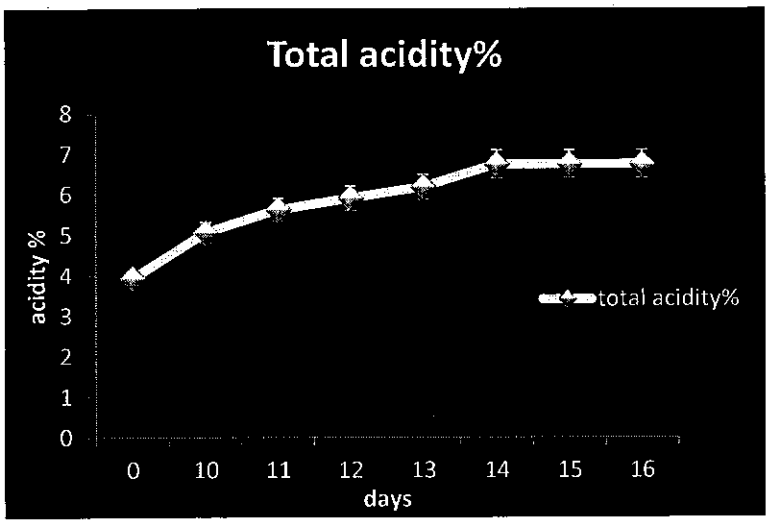
Acids give wines their characteristic crisp, slightly tart taste as well as help in the growth and vitality of yeast during fermentation and protect the wine from bacteria. Alcohol, sugars, minerals, and other components moderate the sourness of acids and give wines balance.

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 [37].



(A)



(B)

FIG 4.15 Monitored analysis for 15 days for total acidity (%) estimation (A) Mahua (1L) setup **(B)** Mahua-guava blend (4L) setup.

- From the graphs it was clear that the % total acidity increased due to blending of mahua must with guava as the mahua product was less acidic, flat heavy and flabby and after blending the product had distinct aroma, sharp taste and crisp mouthfeel due to increase in acidity.
- It also made clear that the pH of mahua-guava fermented product would be lower than that of mahua fermented product.

- Blending of guava must enhanced the aroma, masked the musty odour of mahua product with a fruity smell, increased the content of ascorbic acid and also enhanced the body of the product .

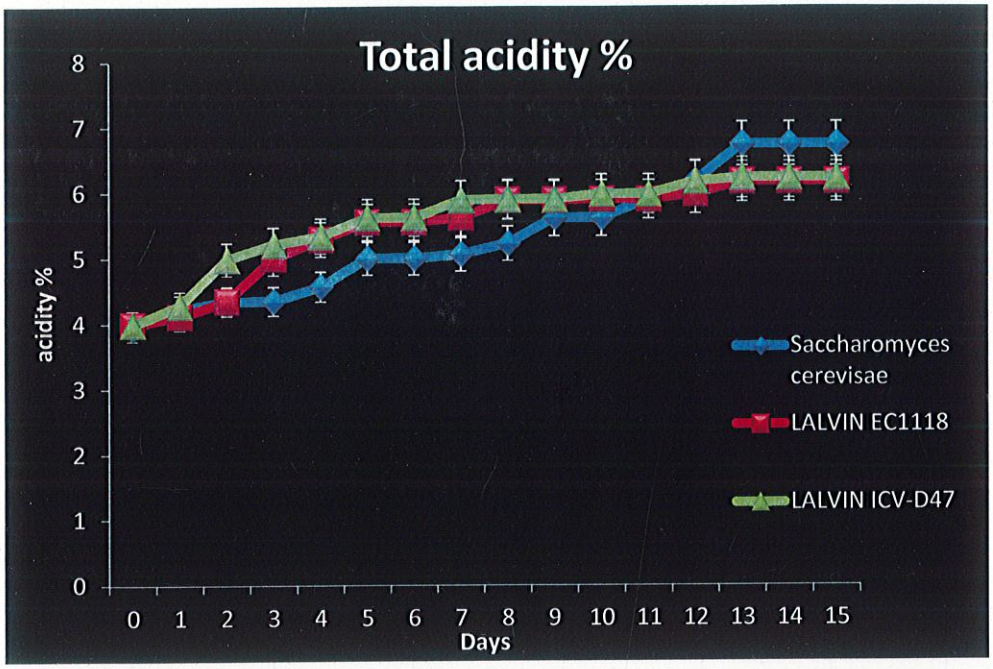


FIG 4.16 Monitored analysis for 15 days for total acidity (%) of mahua-guava blends (1L) with 3 different strains.

- The amount of acidity in a wine determines its balance and is an integral element in its overall structure. Also, an adequate level of acidity can provide protection from certain wine microbes.
- Acidity can play a role in determining how long a wine will age,.
- From the graph it can be stated that the age of product with *Saccharomyces cerevisae* was more followed by the products with LALVIN-ICV D47 and LALVIN EC1118 strains.
- Since all the products had total acidity value more than 6%, they all had a characteristic crisp and slightly tart taste.

d. Higher alcohols estimation.

This is done with the help of a GC-MS.

The volatile composition of wine is based on alcohols, esters and fatty acids and contribute to a characteristic aroma of the fermented product.

Acetone tastes and smells like nail polish remover. Excessive Ethyl Acetate is often formed when wine spoiled with acetic acid reacts with the wine's ethyl alcohol and forms ethyl acetate. Wine with acetone is not organoleptically preferred and is not tasty.

Specefic higher alcohols contribute to the fruity, rose, honey like aroma but at higher concentration they can give rancid or cheezy aroma.

TABLE 4. 1 Biochemical characteristics of fermented mahua (1L) and mahua-guava (4L) product

	Alcohol % v/v	Aldehyde (ppm) as CH_3CHO	Higher alcohols			Acetone %	pH	Esters ppm) as CH3COO C2H5
			C3 % w/w	C4% w/w	C5% w/w			
Mahua (1L) Product	9.1±0.17	48.8±0.7	0.02	<0.01	<0.01	ND	4.23± 0.007	48±0.7
Mahua-guava (4L) Product	8.3±0.16	22.3±0.3	0.02	<0.01	<0.01	ND	3.42± 0.004	56±0.8

- Mahua product had more pH than the mahua-guava product which showed that it was less acidic than the blended product and this statement is already estimated true by the calculation of total acidity %.
- Acetone content was not detected or was negligible this was considered good for the product. So, the fermented product was organoleptically good.
- Lower alcohol content of the product indicated that the product hds a lucrative potential in the market as a low alcoholic nutrabeverage with better aroma and taste.
- Higher alcohols were low in concentration so the products had fruity aroma.

TABLE 4.2 Biochemical characteristics of fermented mahua - guava product with *Saccharomyces cerevisae*

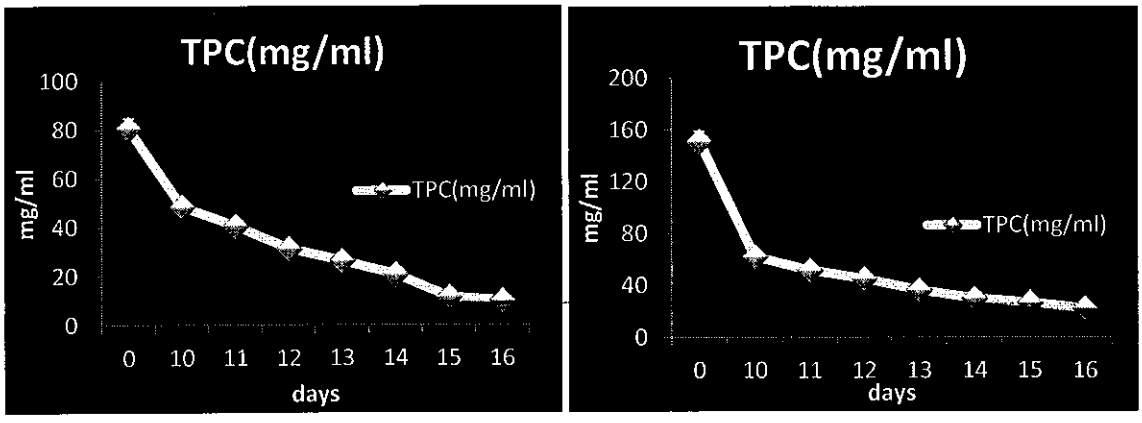
Alcohol % v/v	Aldehyde (ppm) as CH_3CHO	Higher alcohols			Acetone %	pH	Esters ppm) as CH3COOC2H5
		C3 % w/w	C4% w/w	C5% w/w			
6.9± 14	61.1± 0.9	0.02	<0.01	<0.01	ND	3.42 ± 0.004	66 ± 0.9

The mahua-guava fermented product with LALVIN EC1118 and LALVIN ICV-D47 strains were sent for the analysis.

- Lower alcohol content of the product indicated that the product hds a lucrative potential in the market as a low alcoholic nutrabeverage with better aroma and taste.
- Higher alcohols were low in concentration so the products had fruity aroma.
- Acetone content was not detected or was negligible this was considered good for the product. So, the fermented product was organoleptically good.

e. Estimation of TPC (mg/ml)

Here the Folin co-calteu's method was used and calculations were done using the standard plot for TPC (mg/ml).



(A) (B)

FIG 4.17 Monitored analysis for 15 days for total phenolic content (mg/ml) estimation (B) Mahua (1L) setup **(B)** Mahua-guava blend (4L) setup.

- In both the products the TPC value decreased, this showed that polyphenols were adsorbed on the yeast cell wall.

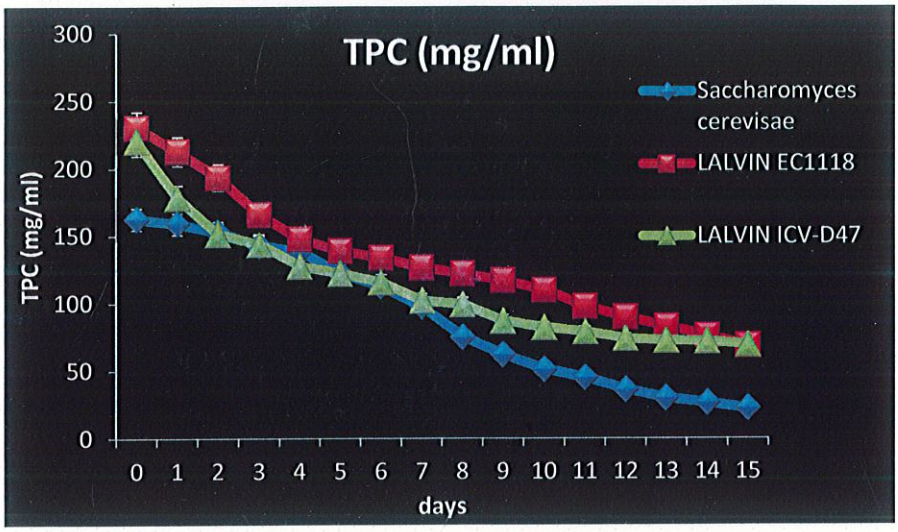


FIG 4.18 Monitored analysis for 15 days for TPC (mg/ml) of mahua-guava blends (1L) with 3 different strains.

- The product that had the highest decrease in TPC was the product with *Saccharomyces cerevisae* strain, followed by the products with LALVIN ICV-D47 and LALVIN EC1118 strains.
- So, the product with LALVIN EC1118 strain had the highest amount of polyphenols.
- LALVIN EC1118 strain can be used to get a fermented nutrabeverage enriched with polyphenols.

4.8 To estimate the amount of total phenolic content (TPC mg/ml) adsorbed by lees and the amount of TPC in fermented product.

The weight of biomass was measured by weighing the dried biomass collected during solid-liquid extraction of TPC and its total phenolic content was measured with the help of an organic solvent (methanol).

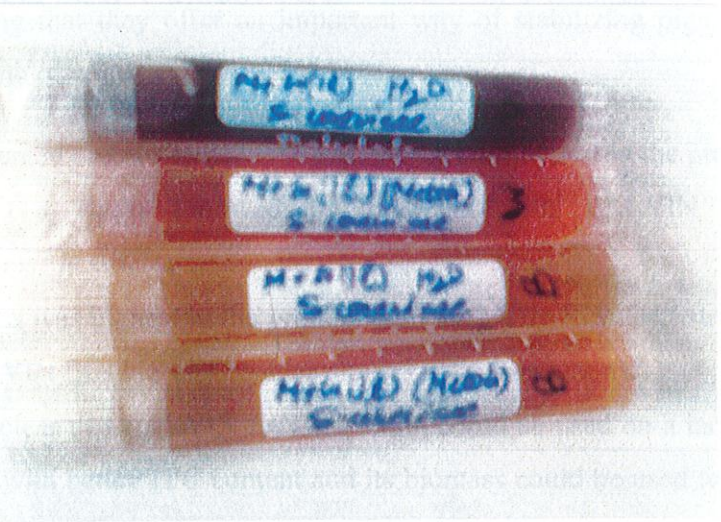


FIG 4.19 Samples for evaluating TPC of wine samples and biomass after extraction with methanol

TABLE 4.3 TPC analysis for different strains after extraction by methanol

	Saccharomyces Cerevisae	**Lalvin EC1118 Strain**	**Lalvin ICV-D47 Strain**
Weight of biomass (gm)	5.9 ± 0.07	13.2 ± 0.09	12.8 ± 0.16
TPC of fermented product(mg/ml) in aq. Solution	57.4 ± 1.87	89.0 ± 0.57	73.9 ± 0.76
TPC of biomass (mg/ml) in aq. Solution	109.7 ± 2.66	138.0 ± 1.42	147.2 ± 1.55

The observed variation in phenolic content after the fermentation may be correlated with several mechanisms- including adsorption of polyphenols onto yeast cell walls, condensation and polymerisation reactions and enzymatic activity [38-40].

During ageing, phenolics evolve and monomeric anthocyanins polymerize by reaction with other flavonoid compounds and aldehydes. It is well known that wine yeasts are among the causes that decrease the phenolic content of wines.

This mechanism can be exclusively physical, involving the establishment of weak and reversible interactions mainly between anthocyanins and yeast walls by absorption . Various yeast metabolites, such as pyruvic acid and acetaldehyde react with different classes of

phenolics, suggesting that they offer an important way of stabilizing pigments during the maturation and ageing of wine [41].

- Here, TPC of fermented product extracted by methanol was highest in the product fermented by LALVIN-EC1118 strain followed by products with LALVIN ICV-D47 and *Saccharomyces cerevisae* strains.
- The TPC of biomass was highest in the product with LALVIN ICV-D47 strain followed by products with LALVIN EC1118 and *Saccharomyces cerevisae* strains.
- From this, its was clear that LALVIN EC1118 strain could be used on a large scale to geta fermented product with better TPC content and its biomass could be used for the extraction of polyphenols for use in food industry.

CHAPTER 5

CONCLUSION

- The category of low alcoholic wines is still small, but showing rapid percentage growth. The market appearance of specially formulated products like McGuigan's "9.5 Chardonnay" and "Finest Denman Vineyard Semillon" at 10.5% alcohol, "Sutter Home Fre Merlot", 1% alcohol, are indications of the rising interest in low-alcohol wine products.
- The standardised methodology has relative technical merits for reducing the ethanol concentration in wine.
- Thus, it can be concluded that mahua flower as a substrate has potential for making good quality fermented product. Addition of guava must improved the flavour of mahua product.
- The blending approaches could be adopted for the improvement of mahua-based fortified products which could solve the problem of low quality traditional beverages by enhancing the nutritional value of the final product, mask the unpleasant flavour of mahua product and improve the texture of the product.
- It can also lead to the production of low- alcohol content beverages with nutraceutical benefits.
- LALVIN EC1118 strain could be used over other strains to get a product rich in polyphenols, sweet flavor and fruity aromas.
- There is a huge scope for product diversification for mahua based drinks and food items.
- Additionally, development of nutrabeverages from mahua flowers could form a good matrix for the therapeutic and nutritionally active constituents and would be a measure of sustainable NTFP management for tribal development.

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