



# **PREPARATION AND QUALITY COMPARISON OF PERRY FROM NEPALI PEARS USING DIFFERENT ISOLATED YEASTS**

**M.Sc. Thesis**

**2018**

**Submitted to  
CENTRAL DEPARTMENT OF BIOTECHNOLOGY  
Tribhuvan University  
Kirtipur, Kathmandu, Nepal**

**A thesis report submitted in partial fulfillment of the requirement for  
M.Sc. degree in Biotechnology**

**Yogendra Chand**



# **PREPARATION AND QUALITY COMPARISON OF PERRY FROM NEPALI PEARS USING DIFFERENT ISOLATED YEASTS**

**M.Sc. Thesis**

**2018**

Submitted to  
**CENTRAL DEPARTMENT OF BIOTECHNOLOGY**  
Tribhuvan University  
Kirtipur, Kathmandu, Nepal

By  
**Yogendra Chand**

*Supervisor:*

**Ganga P. Kharel, PhD**  
Professor of Food technology

Registration number:



**CENTRAL DEPARTMENT OF BIOTECHNOLOGY**

**Tribhuvan University**

**Kirtipur, Kathmandu, Nepal**

Date .....

**Recommendation**

This is to certify that the research work entitled “Preparation and quality comparison of perry from Nepali pears using different isolated yeasts” has been carried out by Yogendra Chand under our supervision.

This thesis work was performed for the partial fulfillment of the Master of Science in Biotechnology under the course code BT 621. The result presented here is his original findings. We, hereby, recommend this thesis for final evaluation.

.....

**Ganga P. Kharel, PhD**  
**Professor of Food Technology**  
**(Supervisor)**



## CENTRAL DEPARTMENT OF BIOTECHNOLOGY

Tribhuvan University  
Kirtipur, Kathmandu, Nepal

Date .....

### Certificate of Evaluation

This is to certify that this thesis entitled "Preparation and quality comparison of perry from Nepali pears using different isolated yeasts" presented to evaluation committee by Yogendra Chand is found satisfactory for the partial fulfillment of Master of Science in Biotechnology.

.....

**Prof. Krishna D. Manandhar, PhD**

Head of Department

.....

**Tika Bahadur Karki, PhD**

Professor of Food technology

Kathmandu University

**External examiner**

.....

**Tribikram Bhattarai, PhD**

Professor

Tribhuvan University

**Internal examiner**

.....

**Ganga P. Kharel, PhD**

Professor of Food technology

**Supervisor**

## **ACKNOWLEDGEMENTS**

I would like to express my sincere thanks to my supervisor Ganga P. Kharel, Professor, for his encouragement, guidance in conducting experiments and enormous co-operation. His guidance has broadened my career prospective and general outlook in life.

I would like to express my sincere thanks to Krishna Das Manandhar, Professor, and Head of the Department, for his motivation and facilitation in my research work. Thanks are also due to laboratory staffs who helped me during my laboratory work.

I would like to dedicate this work to my parents who inspired me, and due to their encouragement I am able to finish this vital goal of my life. They always assisted me during the work in the University and at home. My special gratitude goes to my father for this patience and support during this time. I would also like to thank my entire family who wished my success for this study. I would like to pass gratefulness to my mother who always encouraged me to gain this master degree in Biotechnology.

I am also thankful for the support given by my colleagues, for their assistance and friendship over course of my work. Finally special thanks go to, Bal H. Poudel, for his assistance in pear sample management.

Yogendra Chand

# TABLE OF CONTENTS

	Page
<b>TITLE</b> .....	<b>i</b>
<b>RECOMMENDATIONS</b> .....	<b>ii</b>
<b>CERTIFICATE OF EVALUATIONS</b> .....	<b>iii</b>
<b>ACKNOWLEDGEMENT</b> .....	<b>IV</b>
<b>TABLE OF CONTENTS</b> .....	<b>v</b>
<b>LIST OF FIGURES</b> .....	<b>vii</b>
<b>LIST OF TABLES</b> .....	<b>vii</b>
<b>ABSTRACT</b> .....	<b>viii</b>
<b>CHAPTER ONE</b> .....	<b>1</b>
<b>INTRODUCTION</b> .....	<b>1</b>
1.1 General introduction .....	1
1.2 History of Nepalese wine .....	1
1.3 History of Perry .....	2
1.4 Statement of hypothesis .....	3
1.5 Objectives of the study .....	3
1.3.1 General objectives .....	3
1.3.2 Specific objective .....	3
1.6 Rationale .....	4
<b>CHAPTER TWO</b> .....	<b>6</b>
<b>LITERATURE REVIEW</b> .....	<b>6</b>
2.1 Historical background .....	6
2.1.1 The history of wine production .....	7
2.1.2 Functional aspects of wine .....	8
2.2 Microorganism used in industrial fermentation .....	8
2.2.1 <i>Murcha</i> .....	8
2.3 Fermentation of fruit juice into wine .....	9
2.3.1 Steps involved in winemaking .....	10
2.4 Use of herbs or medicinal plants in wine .....	25
2.5 Analytical techniques for characterizing foodstuffs .....	25
2.6 Classification of wine .....	26
2.7 Introduction to Sensory analysis .....	28
<b>CHAPTER THREE</b> .....	<b>32</b>
<b>MATERIALS AND METHODS</b> .....	<b>32</b>
3.1 MATERIALS .....	32
3.1.1 Equipments .....	32
3.1.2 Chemicals used .....	32
3.1.3 Sampling of murcha, pear and fruits .....	32
3.2 METHODOLOGY .....	33
3.2.1 Microbiological analysis .....	33

3.2.2 Preparation of Perry wine .....	34
3.2.3 Determination of pH, acidity, total soluble solids .....	37
3.2.4 Organoleptic evaluation .....	38
3.2.5 Physicochemical analysis of wine .....	38
<b>CHAPTER FOUR .....</b>	<b>42</b>
<b>RESULT .....</b>	<b>42</b>
4.1 Result .....	42
<b>CHAPTER FIVE .....</b>	<b>52</b>
<b>DISCUSSION .....</b>	<b>52</b>
5.1 Discussion .....	52
<b>CHAPTER SIX .....</b>	<b>60</b>
<b>CONCLUSIONS AND RECOMMENDATIONS.....</b>	<b>60</b>
6.1 Conclusions .....	60
6.2 Recommendations .....	61
<b>REFERENCES .....</b>	<b>62</b>
<b>APPENDIX .....</b>	<b>75</b>

## LIST OF FIGURES

<b>Figure 3.1:</b> Different process involved in the laboratory preparation of Perry wine ...	35
<b>Figure 4.1:</b> Growth of yeast on Sulphur dioxide containing medium .....	44
<b>Figure 4.2:</b> Yeast alcohol tolerance test .....	45
<b>Figure 4.3:</b> Total soluble solid changes of YEPDA broth by wild strain of yeast at 28°C	46
<b>Figure 4.4:</b> Change in pH of fermentable pear juice from yeast isolates .....	46
<b>Figure 4.5:</b> Titratable acidity of fermentable pear juice from yeast isolates .....	47
<b>Figure 4.6:</b> Change in °BRIX of fermentable pear juice from yeast isolates .....	48
<b>Figure 4.7:</b> Sensory evaluation of commercial wine and lab produced wine .....	48
<b>Figure 4.8:</b> Chromatogram of standard volatile mix .....	50
<b>Figure 4.9:</b> Chromatogram of commercial wine .....	50
<b>Figure 4.10:</b> Chromatogram of wine volatile fermented by yeast isolated from Palpa's murcha .....	51
<b>Figure 4.11:</b> Chromatogram of wine fermented by yeast isolated from ghanti phool flower <i>Argyrea nervosa</i> .....	51

## LIST OF TABLES

<b>Table 4.1:</b> Total number of yeasts obtained in YEPD agar plates obtained by colony count method .....	42
<b>Table 4.2:</b> Study Morphological characteristics of the isolates .....	42
<b>Table 4.3:</b> Growth at different sugar concentration .....	43
<b>Table 4.4:</b> Report on different parameters and chemical compounds present in the different variety of wine .....	49

## ABSTRACT

Local starter *murcha*, *ghanti phool* (*Argyrea nervosa*) and fruits such as raisin, grape, apricot were selected for the isolating wine yeast capable of efficient fermentation. The *murcha* were collected from places like Jorpati, Palpa, Lubhu, Sunsari, Bhojpur, *ghanti phool* was obtained from Kritipur and fruits were purchased from local market. Yeast extract peptone dextrose (YPD) agar medium with 50 ppm chloramphenicol was used as a substrate to isolate yeast strains. The nine yeast strains isolated were screened for their tolerance in sugar, sulphur dioxide, alcohol and checked for their alcohol production capacity. All isolated strains exhibited growth in YPD broth containing 20% dextrose, 14% alcohol and 75 ppm Sulphur dioxide. However, yeast from *murcha* (Palpa *murcha*), and *ghanti phool* flower exhibited the best result and these two isolates were selected for fermenting pear juice. Extracted juice from pear fruit was ameliorated to 20° Brix. The ameliorated juice was pasteurized (65°C for 5min), inoculated with yeast and held at room temperature for 20 days. Addition of pectinase solution at the concentration of 5 ppm showed the best result as compared 600 ppm bentonite, 6% dry gelatin, 0.15% egg white. Soluble solids, pH decreased while titratable acidity increased with increasing length of time. The highest alcohol production was shown by wine produced using flower yeast (W-YFW) 10.5% compared to the wine produced using commercial yeast (W-SN9) 9.17% and wine produced using Palpa yeast (W-YPM) 9.84%.

Result of sensory evaluation conducted by the untrained panelist showed that there was similar character in context of flavor, taste, and clarity but had some difference in color and aroma between the labs prepared pear wine and reference wine. The overall acceptability of the pear wine prepared in the lab was similar with the reference wine. Three lab wine prepared W-YPM, W-YFW AND W-SN9 and four commercial wines C.W-1, C.W-2, C.W-3 and C.W-4 were subjected to quantitative chemical analysis for the presence of the titratable acidity, volatile acidity, esters, total phenol, alcohol percentage, SO<sub>2</sub> free and total. pH, °Brix, titratable acidity (%MA), volatile acidity (g/L), ester (mg/L), total phenol (mg/L), free SO<sub>2</sub> (mg/L) and total SO<sub>2</sub> (mg/L) of all wines ranged from 3.34 to 3.72, 5.8 to 13.8, 0.29 to 0.48, 0.111 to 70 to 281, 53 to 110, 2.30 to 5.12, 2.30 to 32.64 respectively.

Gas Chromatography Qualitative analysis of the lab prepared wine using yeast isolated from Palpa *murcha*, flower and commercial wine samples against control showed the presence of the acetaldehydes, acetone, ethylacetate, ethanol, methanol, propanol-2 and propanol-1. The methanol limit on usage is often set at 250 mg/L with an alcoholic strength by volume of less than 15% by International Organization for Vine and Wine (OIV), however presence of in the commercial wine did not exceeded the limit and was

found to be 130mg/L. Quantitative analysis of three wine samples showed the percentage of acetaldehydes, acetone, ethanol, methanol and propanol-2 between 0.004 to 0.041, 0.003, 9.3 to 12.3, 0.013, and 0.003 to 0.0019 respectively.

**Keywords; *Marcha*, Pear, Yeast, Fermentation and Clarification.**

# **CHAPTER-ONE**

## **INTRODUCTION**

### **1.1 General introduction**

Wine is fermented grape juice containing varying percentages of alcohol together with polyphenols, ethers and esters that give it bouquet and flavor. Although the juice of other fruits, berries, and vegetables can be fermented to create alcohol, fruit wines are generally qualified by the name of the produce used, such as gooseberry wine and blueberry wine. The word “wine” when used alone refers to an alcoholic beverage made from grapes. Wines come in various colors (red, white, and rose) and many types, which include dry and sweet, still and sparkling, and wines fortified with grape spirit (brandy). There are also many wine-based drinks, such as wine coolers and offering peach, kiwi, and strawberry wines. There are many different styles of wine, allowing wine to satisfy a wide range of individual tastes and occasions and permitting wine to accompany many styles of food. Most table wines are dry in the technical sense that they contain no residual sugar because all the sugar that was in the grapes (or added to the must) has been fermented out. Even so, wines can feel sweet in the mouth because of their fruit flavors, and many varietals such as chardonnay, Shiraz, and zinfandel have a sweet fruit dimension to them (Saranraj et al., 2013).

Fruit wines are fermented alcoholic made from a variety of base ingredients (other than grapes) they may also have additional flavors taken from fruits, flower and herbs. Fruit wine have traditionally been popular with home winemakers and in areas with cool climates such as North America and Scandinavia. Fruit wine can be made from virtually any plant matter that can be fermented. Most fruits and berries have the potential to produce wine. There are a number of methods of extracting flavor and juice from the fruits or plants being used, pressing the juice, stewing and fermenting the pulp of the fruits are common. Few foods other than grapes have the balanced quantities of sugar, acid, tannins, nutritive salts for yeast feeding and water to naturally produce a stable, drinkable wine. However, some of these products do require the addition of sugar or honey to make them palatable and to increase the alcohol content. The amount of fermentable sugar is often low and needs to be supplemented by a process called chaptalization in order to have sufficient alcohol levels in the finished wine (Harding, 2005).

### **1.2 History of Nepalese wine**

History of Nepalese wine almost started in the year 1995 A.D when Aish Narayan Shrestha started making wines, using the wild berries that grow above 8,000 feet in the eastern hilly region of Nepal. He organised villagers in Sankhuwasabha to collect aiselu and chutro in the

forests, and then sell it to his factory. Then the berries were mixed with sugar and left to ferment in plastic drums for a month. Hinwa is entirely dependent on a natural process. However the company then had improvised Nepali distillation process and not adopted modern distillery technology. Hinwa winery produces white and red wines, although the red is closer to rose. Production was severely hit when the Maoists destroyed their factory in 2001 for producing alcohol. But Hinwa pulled itself together and has quietly gained popularity among wine drinkers in Nepal. The majority of Nepalese wine is made from a combination of fruits and herbs. The most common fruits found in Nepalese wines are small yellow raspberries (known here as *aiselu*), and Himalayan barberries (*chutro*).

Many brands of Nepal made wines are available in the market and have been able to capture significant beverage value of the market. Within the short period of time of about five years, the consumers of Nepali wines have grown significantly. The taste and high qualities of Nepali wines have been able to pull the consumers towards them. Some of the popular brands of wines made in Nepal are mentioned below:

#### **Dadaghare**

The wine manufactured in *Pokhara, Dadaghare* is considered to be among the first Nepali wine. It is not only popular among the local customers but also foreigners. The wine available in four different flavors and is manufactured using various fruits, herbal fruits and honey and is absolutely chemical free.

#### **Nettlange**

Manufactured by *Sakaro Beverages, Nettlange* is one of the popular Nepali wines in the local market. Made from nettles (*Sishno*) and oranges.

#### **Grapple**

Made from black grapes that are imported from India, and apples from *Mustang, Grapple* is manufactured by *Sakaro Beverages*.

#### **Divine**

One of the fast selling brands available in the market, Divine wine was introduced in 2010. The wine manufactured by *Shree Mahakali wine*, it is made of grapes, spices, tea and various other fruits.

### **1.3 History of Perry**

France was the first country to have produced Perry after the collapse of the Roman Empire. In the medieval period the majority in the West of England were imported from France. However, due to the favorable condition of the climate and soil in England they started pear cultivation. Thereafter Perry making became the well-established company in England between sixteenth and seventeenth century. Perry production reached its zenith of popularity during the eighteenth century after the English Civil War had begun, when the

large number of soldiers in the country became acquainted with it and at the same period conflicts with France made the importing of wine difficult. This led many farmers and estates of England to cultivate pear in their own orchards (Wilson, 1993).

Pear trees lengthy time to mature and intensive labor to crop resulted in the decline of production of traditional Perry in the 20<sup>th</sup> century. Francis Showering innovation in the Perry making techniques revived by adding sparkling appearance to Perry and named it Babychan. Babychan, the first mass- produced branded Perry, was developed by Showering from the application of the long Ashton Institute's research using authentic Perry pears. At present Perry is produced from pear concentrate. A competing brand of commercial Perry such as Lambrini, Irish, are available in the world market. However, Perry has in recent time increased in popularity among British consumers. Now Pear fermented beverages are produced in countries such as, Australia, Sweden, United States, New Zealand, Japan and Austria. Pear Cider is an alcoholic drinks containing pear juice, although industrially made from pear concentrate. According to the National Association of Cider Makers the terms Perry and Pear cider are interchangeable.

## **1.4 Statement of hypothesis**

This research work was based on 3 main hypothesis which are listed below:

1. *Murcha* has been used as indigenous starter to ferment locally produced beverages in Nepal. So the *murcha* could contain high potential wine yeast which might impart good flavor in the wine prepared.
2. *Flower ghanti phool (Argyrea nervosa)* as well as fruits such as grapes, raisin, apricot are rich sources of naturally present sugar. This sugary atmosphere could support adequate environment for the fermentable microbes to live.
3. Pear fruits of Pharping, Kritipur area are known for its uniformity in the quality, size, taste and aroma. Ripened fruits contain high sugar and its possibility to use as raw material for wine making may prevent its losses.

## **1.5 Objectives of the study**

### **1.5.1 General objective**

The general objective of the research was to prepare Nepali pear's wine (perry) from the yeast isolated from indigenous starter culture (*murcha*).

The specific objectives of this research work were:

1. Screening and isolation yeasts from various *murcha*, fruits and flower samples of Nepal.
2. Evaluation of fermentative performance of the yeasts.

3. Production of perry from the Nepali mountain pear grown in Pharping of Kirtipur pear juice by submerged state fermentation.
4. Assessment of the quality of the wine based on pH, acidity, total soluble sugar, alcohol contents, volatile acidity, methanol, aldehydes, ester, and total and free SO<sub>2</sub>.

## **1.6 Rationale**

Pharping area of Kirtipur sub-metropolitan city has been known for producing Nepali mountain pear. This region of Nepal has suitable climate and possibility of pear farming. In Nepal and the Himalayan states of India; they are cultivated as a cash crop in the Middle Hills because of its climate suitability. The fruit are carried to nearby markets by human porters or, increasingly, by truck. Pear fruit is strong than other fruits as it grows without any disease problems. Pear farming in Nepal started since ancient time, popularity of pear farming has been increasing rapidly between Nepali farmers. Now farmers are interested in growing and preserving these indigenous pear trees in hilly region of Nepal.

Pear fruits produced by many indigenous trees are edible and can ripen within a very short span of time, generating surplus production. Many of these pear fruits are delicious, juicy and consumed fresh, but large quantities are wasted during peak harvest periods, due to high temperature, humidity fluctuations, improper handling. Directly after harvest, cooling is essential before marketing. Pre-cooling is the removal of field heat directly after harvest before any further handling. Any delays in pre-cooling will shorten postharvest shelf life and reduce quality. Fruit deteriorates at a slower rate than that has not been cooled. In Nepal, there is not such practice for pear. Long term storage of pear is not common practice in Nepal. At wholesale market, jute or gunny sacks, plastic crates, bamboo baskets with fruits are kept upright positions in single stake. At wholesale market, pear is stored for maximum of 3-4 days but at retail shop, it is stored for 1-2 days. Postharvest loss of Asian pear due to skin blackening followed by decay. Spores on bins/gunny sacks/bamboo baskets or fruit surface itself are the source of fungal diseases appeared in the storage or packinghouse. Orchard sanitation is essential to reduce postharvest disease in store. Using clean harvesting packs such as gunny bags and bamboo baskets helps to reduce postharvest diseases. Wounds are the primary entry site for postharvest decay. Minimize wounds when handling fruits. Senescent scald is appeared in pear as brown to black discoloration on the skin of the fruit and the flesh is not initially affected. Superficial scald develops rapidly after fruits have been ripened or warmed.

The food industries are using variety of preservation and processing methods to extend the shelf life of fruits and vegetables such that they can be consumed year round and transported safely to consumers all over the world, not only those living near the growing

region. Therefore, utilization of ripe fruits or their juices for wines production is considered to be an attractive means of utilizing surplus and over ripen fruits. Moreover, fermentation helps to preserve and enhance the nutritional value of foods and beverages. The research underway currently is to assess the potential of fruit species which have been explored by the food industries to meet the growing needs of the ever-increasing consumer market for several fruits by-products including wines.

Fermentation is the best way to add value of the produce and requires very little sophisticated equipment, either to carry out the fermentation or for subsequent storage of the fermented product. It is a technique that has been employed for generations to preserve fruits in the form of drinks and other food for consumption at a later date and to improve food security. Basically, most fruits can be fermented if not all provided they are well prepared. It is, therefore, a highly appropriate technique for use in developing countries and remote areas where access to sophisticated equipment is limited. Fermented fruit wines are popular throughout the world, and in some regions, it makes a significant contribution to the diet of millions of individuals. Harvested fruits may undergo rapid deterioration if proper processing and storage facilities are not provided, especially in the humid tropics where the prevailing environmental conditions accelerate the process of decomposition. Although, there are several options for preserving fresh fruits, which may include drying, freezing, canning, and pickling, and many of these are inappropriate for the product and use on small scale in developing countries. For instance, the canning of fruits at the small-scale has serious food safety implications and contamination, especially botulism. (Sarah, 2017).

This research is a means to produce a pear wine and make it available in the local market to the best of its quality. So far research on pear wine has not been carried out until now. Within the short period of time of about a decade, it has been observed that wine functional properties have attracted the Nepalese beverage consumer significantly. In order to fulfill the demand, wine industries in the country are rising in number and spend money mainly in substrates imported from other country, if homely produced pear is used as substrate for the production of beverages it will definitely uplift the nation economy, reduce the dependency on the neighboring country, create employment to the youth, attract the tourist for its uniqueness and will make Nepal a prosperous country to be really admired at.

# **CHAPTER TWO**

## **LITERATURE REVIEW**

### **2.1 Historical background**

Fermentation is one of the oldest forms of food preservation technologies in the world. Indigenous fermented foods such as bread, cheese, and wine have been prepared and consumed for thousands of years and are strongly linked to culture and tradition, especially in rural households and village communities. The development of fermentation technologies is lost in the midst of history. Anthropologists have postulated that it was the production of alcohol that motivated primitive people to settle down and become agriculturists. Some even think that the consumption of fermented food is pre-human (Hornsey, 2012).

The first fermented foods consumed probably were fermented fruits. Hunter-gatherers would have consumed fresh fruits but in times of scarcity would have eaten rotten and fermented fruits. Repeated consumption would have led to the development of the taste for fermented fruits. There is reliable information that fermented drinks were being produced over 7000 years ago in Babylon, 5000 years ago in Egypt, 4000 years ago in Mexico, and 3500 years ago in Sudan (Yokotsuka, 2015). There is also evidence of fermented meat products being produced for King Nebuchadnezzar of Babylon. China is thought to be the birthplace of fermented vegetables, and the use of *Aspergillus* and *Rhizopus* molds to make food. The book called “Shu- Ching” written in the Chou dynasty in China (1121–256 BC) refers to the use of “chu” a fermented grain product (Dirar, 1993).

Knowledge about traditional fermentation technologies has been handed down from parent to child, for centuries. These fermented products have been adapted over generations; some products and practices no doubt fell by the wayside. Those that remain today have not only survived the test of time but also more importantly are appropriate to the technical, social, and economic conditions of the region. According to Robinson, (Robinson, 2016) Natural occurrence of fermentation means that it was probably first observed long ago by humans. The earliest uses of the word “Fermentation” in relation to winemaking were in reference to the apparent “boiling” within the must that came from the anaerobic reaction of the yeast to the sugars in the grape juice and the release of carbon dioxide. The Latin “fervere” literally means to boil. In the mid-19th century, Louis Pasteur noted the connection between yeast and the process of the fermentation in which the yeast acts as catalyst through a series of a reaction that converts sugar into alcohol. The discovery of the EMP pathway by Gustav Embden, Otto Fritz Meyerhof, and Jakub Karol Parnas in the early

20th century contributed more to the understanding of the complex chemical processes involved in the conversion of sugar to alcohol.

### **2.1.1 The history of wine production**

Wine has been produced for thousands of years. Wine is a distinctive product that influences major life events, from birth to death, victories, auspicious occasions, harvest, and other events, due to its analgesic, disinfectant, and profound mind-altering effects (McGovern & Zhang, 2004).

Once upon a time, many years ago a man found a closed fruit jar containing a honey bee. When he drank the contents, he tasted a new, strange flavor. Suddenly his head was spinning he laughed for no reason, and he felt powerful. He drank all the liquid in the jar. The next day all he experienced was an awful feeling. He had a headache and an unpleasant taste in his mouth and dizziness he had just discovered the hangover. You might think this is just a tale, but is it? Several archaeological excavations have discovered jars containing the remains of wine that are 7000 years old (McGovern, 2009) and it is very likely that humankind's first encounter with alcoholic beverages was by chance.

Over the course of human history, and using a system of trial and error and careful observation, different cultures began producing fermented beverages. Mead, or honey wine, was produced in Asia during the Vedic period (around 1700-1100 BC), and the Greeks, Celts, Saxons, and Vikings also produced this beverage. In Egypt, Babylon, Rome, and China, people produced wine from grapes. In North America, people produced chicha from grains or fruits. While in North America, people made octli (now known as "Palque") from agave, a type of cactus (Godoy et al., 2003).

At the time, people knew that leaving fruits and grains in covered containers for a long time produced wine and beer but no one fully understood why the recipe worked. The process was named fermentation from the Latin word *fervere* which means "to boil". The name came from the observation that mixtures of crushed grapes kept in large vessels produced bubbles, as though they were boiling. Producing fermented beverages was tricky. If the mixture did not stay long enough, the product contained no alcohol, and if it left for too long the mixture halted and was undrinkable. Through empirical observation, people learned that temperature and air exposure are key to the fermentation process. Wine producers traditionally used their feet to soften and grind the grapes before leaving the mixture to stand in buckets. In so doing, they transferred microorganisms found on their feet into the mixture, at the time, no one knew that the alcohol produced during fermentation was produced because of one of these microorganisms. A tiny one-celled eukaryotic fungus that is invisible to the naked eye; yeast. It took several hundred years before quality lenses and microscopes revolutionized science and allowed researchers to observe this microorganism.

## **2.1.2 Functional aspects of wine**

Wine consists of a diverse commodity class composed of the yeast fermentation products of must (or fruit juice). Wine is a fruit product, but fermentation produces a variety of chemical changes in the must and so wine is far from being juice with ethanol added. Both clinical and experimental evidence suggest that moderate consumption of red wine offers greater protection to health by reducing cardiovascular morbidity and mortality and this is attributed to antioxidant polyphenolics other than alcohol which is found particularly in red grape wine (Halpern, 2008). The phenolic acids can scavenge free radicals and quench reactive oxygen species and therefore provide effective means of preventing and treating free radical-mediated diseases (Gresele, 2011). Furthermore, wine polyphenols can lead to the modulation of both oral and gut microbiota (Requena & Monagas, 2015).

## **2.2 Microorganism used in industrial fermentation**

### **2.2.1 *Murcha***

*Murcha* or *Marcha* are the indigenous starters that are used to ferment the locally produced beverages in Nepal. These are known by different names according to ethnic groups and mostly prepared by Rai, Limbu, Tamang, Gurung, Newar, and Tharu communities in Nepal. With the long history of the existence of these starters, there is no evidence of the time it gets started. The ingredients and process of making starters is kept secret that not daughters, only daughters-in-law are taught.

This starter culture is a mixed type which comprises various saccharifying molds, fermentative yeast, and acidifying lactic acid bacteria where the loads were found to be  $10^6$  CFU/g,  $10^8$  CFU/g, and  $10^7$  CFU/g respectively (Tamang, 1995).

Although various groups of microorganisms are present, selective growth of desirable microflora is crucial that includes *Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera*, *Clavaria versatilis*, *Rhizopus* sps and *Pediococcus pentosaceus*. The two main types of *marcha* are Mana and Manapu.

### **Traditional methods of *Murcha* preparation in Nepal**

The general method of preparation involves soaking of rice (*Oryza sativa*) in water for 8-10 hours and crushing into fine powder. Mixing of different ingredients, like roots, leaves, and flowers from various *murcha* plants, ginger, dry red chili and previously prepared *murcha* is performed. Water is added to the mixture and kneaded into cakes of different shape and size. Then, they are kept for fermentation for 1-3 days. The distinctive aroma and swollen structure of cakes specify the end of fermentation which is sun-dried for next few days.

### **1. Plants used in preparation of *murcha***

Among the 42 plants known to be used in preparation of *murcha*, depending on the place where they are prepared different plants such as Byancha flower (*Antirrhinum*), Chito (*Plumbago zelanica*), Mulapate (*Dipsacus mitis*), leaves of Guava (*Psidium guajava*), Gaitchare (*Inula cappa*) and leaves of Jack fruit (*Artocarpus integra*) are used. Since identify of these plants are mostly kept secret by local people. The use of plants in *murcha* is based on the specific ethnic groups and distribution of plants (Shrestha, 1991).

### **2. Preparation of *Manapu* starter**

The traditional technology of *manapu* starter is kept so secret by some people of lubhu area in Kathmandu valley, that it is not even taught to daughters but only to the daughter-in-law because of fear of exposition of secret.

*Manapu* plants and dried flowers are added in rice flour and kneaded with water until the uniform dough is prepared. Then, it is molded into round cakes of 4-6cm in diameter and 1-3cm thick, which is dusted with powdered cake from previous batch.

Thus, prepared cake is spread over a bed of paddy straw, and few consecutive layers are made which is again covered with straw mat (*sukul*) and are allowed to ferment for 7-8 days after which the yeast cakes (*murcha*) are sun dried stored (Karki, 1986).

### **3. Preparation of *Mana* starter**

Cleaned wheat is steamed until the grain ruptures and become lumpy. Then the steamed treated wheat is dusted with *mana* of previous batch and spread over a bed of paddy straw, and few consecutive layers are made, covered with straw mat (*sukul*) and are allowed to ferment for 6-7 days after which, fermented wheat appear as greenish granule. Thus prepared *mana* is sundried and stored in earthen jar (*Ghyampo*).

## **2.3 Fermentation of fruit juice into wine**

Wine fermentation is one of the most ancient of human's technologies and is now one of the most commercially prosperous biotechnological processes (Arribas & Polo, 2004). Fruit juices are fermented to produce wine, an alcoholic beverage. Grapes are usually preferred because of the natural chemical balance of the grape juice which aids their fermentation process without the addition of sugars, acids, enzymes, or other nutrients. However, fruits such as apple, pear, strawberry, cherries, plum, banana, pineapple, oranges, cucumber, watermelon, guava, cucumber, pineapple, and other fruits are used in wine production (Obaedo & Ikenebomeh, 2015). Using species of *S. cerevisiae* which converts the sugar in the fruit juices into alcohol and organic acids, that later react to form aldehydes, esters, and other chemical compounds which also help to preserve the wine (Fleet, 2013).

### **2.3.1 Steps involved in Winemaking**

Winemaking involves mainly following steps:

- a. Raw materials
- b. Juice extraction
- c. Pre-treatment
- d. Fermentation
- e. Clarification
- f. Maturation

#### **a. Raw materials for wine preparation**

##### **Wine yeast**

Yeasts are of great economic importance. Yeasts, especially, different strains of *S. Cerevisiae*, have long been used for the production of alcoholic beverages, solvents, and other chemicals. Yeast is a unicellular fungi or plant-like microorganism that exists in or on all living matter, i.e., water, soil, plants, and air. They are a microbial eukaryote, associated with Ascomycetes, and are rich in protein and Vitamin B (Dunn et al., 2015). Yeasts flourish in habitats where sugars, water, warmth and nitrogenous material are present, such as fruits, flowers, and bark of trees. However, commercial yeasts of today are quite different from wild strains due to genetic manipulation, allowing them to grow in previously unsuitable conditions (Kumar et al., 2015). More than 8,000 strains of this vegetative microorganism have been classified. About 9–10 pure strains with their sub classifications are used for the fermentation of grain mashes. These belong to the type *S. cerevisiae*. Each strain has its own characteristics and imparts its special properties to a beverage when used in fermentation. A limited number of yeasts in the classification *Saccharomyces ellipsoides* are used in the fermentation of wines from which brandy is distilled. Since yeasts function best in slightly acid medium, the mash, juice, sap, or extract prepared for fermentation must be checked for adequate acidity.

Yeasts are the principle organisms involved, filamentous fungi, lactic acid bacteria, acetic acid bacteria and other bacterial groups all play a role in the production of alcoholic fruit products. Normal grapes harbour a diverse micro flora, of which the principle yeasts (*Saccharomyces cerevisiae*) involved in desirable fermentation are in the minority. Lactic acid bacteria and acetic acid bacteria are also present. The proportions of each and total numbers present are dependent upon a number of external environmental factors including the temperature, humidity, stage of maturity, damage at harvest and application of fungicides. It is essential to ensure proliferation of the desired species at the expense of the non-desired ones. This is achieved through ensuring fermentation conditions as such to encourage *Saccharomyces* species (Bajaj et al., 2011).

The fermentation may be initiated using a starter culture of *Saccharomyces cerevisiae*, in which case the juice is inoculated with populations of yeast of  $10^6$  to  $10^7$ cfu/ml juice. This approach produces a wine of generally expected taste and quality. If the fermentation is allowed to proceed naturally, utilizing the yeasts present on the surface of the fruits, the end result is less controllable but produces wines with a range of flavor characteristics. It is likely that natural fermentations are practiced widely around the world, especially for home production of wine. In natural fermentations, there is a progressive pattern of yeast growth. Several species of yeast, including *Kloeckera*, *Hanseniaspora*, *Candida* and *Metschnikowia* are active for the first two to three days of fermentation. The buildup of end products (ethanol) is toxic to these yeasts and they die off, leaving *Saccharomyces cerevisiae* to continue the fermentation to the end. *Saccharomyces cerevisiae* can tolerate much higher levels of ethanol (upto15%v/v or more) than the other species who only tolerate up to 5% or 8% alcohol (Aidoo, 1994). Because of its tolerance of alcohol, *Saccharomyces cerevisiae* dominates wine fermentation and is the species that has been commercialized for starter cultures (Adams, 1990).

## **Fruits**

Grapes are the most common fruit used as raw material for alcoholic fermentation. Historically, wine is the product of fermentation of grape species *Vitis vinifera*. The high sugar content of most *V. vinifera* varieties at maturity is the major factor in their selection for use in much of the world's wine production. Their natural sugar content provides the necessary material for fermentation. It is sufficient to produce a wine with an alcohol content of 10 percent or higher. Wines containing less alcohol are unstable because of their sensitivity to bacterial spoilage. The grape's moderate acidity when ripe is also favorable to wine making. The fruit has an acidity of less than 1 percent, calculated as tartaric acid, the main acid in grapes, with a pH of 3.1 to 3.7. The flavor of grapes varies from neutral to strongly aromatic, and the pigment pattern of the skin varies from light greenish-yellow to russet, pink, red, reddish violet, or blue-black. Grapes also contain tannins needed to give bite and taste in the flavor of wines and to protect them from bacteria and possible ill effects if overexposed to the air.

Other fruits can be used to produce wine. When fruits other than grapes are used, the name of the fruit is included, as in papaya or pineapple wine. Apples and citrus fruits with sufficient fermentable sugars are crushed, and the fermentable juices are either pressed out for fermentation or the entire mass is fermented. Tropical fruits such as guava, mangoes, pineapple, pawpaw, ripe banana, ripe plantain, tangerine, and cashew fruit also contain fermentable sugars with levels varying from 10 to 20 percent (Kuboye et al., 1978).

## Pear

Pear is the second most important deciduous fruit in Nepal. In Nepal, they are cultivated as a cash crop in the Middle Hills between about 1,500 and 2,500 meters' elevation where the climate is suitable. Pear plantation covers 4,485 hectare with a total production and productivity of 34,231.4 mt and 9.93 mt per ha respectively (MoAD, 2014). There are two kinds of pear grown in Nepal. European pear (*Pyrus communis*) and Asian pear (oriental/sand pear -*Pyrus pyrifolia* or *P. serotina*). The popular germplasm of sand pear in Nepal is Pharping Sthaniya which is very popular in mid-hills due to its excellent fruit quality and high yield. Some important cultivars of oriental pear are introduced into Nepal during the Horticulture Development Project Phase I (1985-1990) with the technical support of the government of Japan (JICA). These cultivars include Hosui, Kosui, Shinko, Chojuro, Shinsui and Okusankichi. Studies on Pharping Sthaniya showed that there were no differences in tree form and fruit quality (Sakuma, 1995).

European cultivars are also introduced and evaluated for temperate regions of Nepal. These cultivars require very careful management and high chilling units (>800-1000 hours), and can be grown in cool temperate region. The better adapted European pear cultivars in Nepal are Bartlett, Quince, Anjou and Conference (Devkota, 2000).

### Cultivation system and postharvest operations

Pear is mainly grown in upland conditions in Nepal. Farmer's plant intercrops so long as there is sufficient space available between trees. It is grown in rainfed conditions and is not generally irrigated. However, where irrigation facility is available, trees are irrigated after fertilized in winter, and drought period during March-June. In Pharping and Chhaimale villages of Kathmandu, most of the orchards are found to be over branched and overloaded with many small fruits. Fruits arrive in the market from mid-July to end of September. Flesh firmness, colour change, sweetness, ease of separation of stalk from spur are the major maturity indices. Maturity of pear at harvest determines the length of time the fruit can be stored. Skin of Pharping Sthaniya is brown as expected to be good.

Pear harvesting solely done manually in Nepal. Asian pear is harvested two to three times from July to September in Nepal. For Pharping Sthaniya, the fruits can be harvested when the penetrometer reading ranges from 6-7 kgF (kilogram Force). However, in general, fruit firmness is not a good indicator of maturity for Asian pear. Color and taste are better maturity indices for Asian pear. Harvesting of the fruits is carried out after the dew or rain drops disappeared on the fruit surface, for example, late morning and evening are appropriate times of the day. Fruits are picked by hand and are collected in one place in field under the tree or shed house before they are packed in gunny sacks or bamboo baskets (Doko or Thumse). Badly damaged fruits are sorted out before packing. Fruits are packed in

bamboo baskets/ jute/plastic sacks each weighing about 45-50 kg and transported by porters to nearby markets.

#### Uses

Pear is mostly used as table purpose and eaten raw. It is popular for its juicy, crispy and sweet taste and texture. Asian pears are also known as apple“-pears” because of their apple-like texture. It is freshly eaten with or without other fruits. Sometimes it is used as fruit salad mixed with yogurt and added sugar. It is used to prepare juice, preserved juice or pear pie and wine in Nepal.

#### Total solids (TSS)

Total soluble solid is a classical standard method for maturity determination for pear as many fruits. TSS increases as approaching maturity. Asian pear should be harvested when TSS reaches at least 12-13%.

#### Titrateable acid (TA)

Total acid in pear affect taste and flavor but is a poor prediction of fruit maturity. Titrateable acid level varies with year and orchard conditions. TA in Asian pear is one of the better maturity indices and is measured to determine the right harvesting stage. Predominant acid of pear is malic acid.

#### Asian pear

Fruit commonly known as the Asian pear in different parts of the world include: Asian pear, Chinese pear, Korean pear, Japanese pear, Taiwanese pear, and sand pear. *Pyrus pyrifolia*, called Chinese pear or Nashi pear, usually round, with brown or yellow skin. *Pyrus pyrifolia* is a species of pear tree native to East Asia. *Pyrus bretschneideri*, called Ya pear or Chinese white pear, usually slightly elongated (shaped like a European pear), with yellow skin (Bailey et al, 1976).

In Korea, the fruit is known as *bae*, and it is grown and consumed in great quantity. In the South Korean city of Naju, there is a museum called The Naju Pear Museum and Pear Orchard for Tourists. In Australia, these pears were first introduced into commercial production beginning in 1980 (NSW Primary Industries, 2002).

## **b. Juice extraction**

There are several methods to extract juice depending on the type of fruit used. For citrus fruits which are naturally juicy, the best option is to use a hand presser or a revolving citrus 'rose'. Some fruits such as melon and papaya are steamed to release the juice. Apples are pressed and fruits such as mango, guava, pineapple, strawberry must be pulped to extract the juice. The fruit pieces are pushed through a perforated metal plate that crushes and turns them into a pulp. Some fruits can be pulped in a liquidizer and then filtered to remove

the fruit pieces. There is a range of equipment available that varies in size and in the type of power supply (Ali, 2008).

Presses are the usual and traditional method of removing juice from fruit and vegetable materials. However, recently diffusion extraction, centrifugation, and specialized ultrafiltration techniques have been explored and have been exploited commercially to a limited extent. Yield efficiency diagrams that relate juice yields to mash feed rates provide a mechanism for comparing presses and other processes such as enzyme treatments or decanter centrifuges for efficiency under a stated set of circumstances. Diffusion extraction is capable of removing 90 to 94% of soluble solids from properly prepared apple slices, but the resulting juice is diluted with extraction water and is high in extracted tannins. Concentration is necessary to obtain juice solids equivalency, and the resulting juice has sour/astringent flavors that must be removed with tannin absorbants to provide acceptable flavor. Currently, decanter centrifuges are used commercially and have provided an alternative to presses under certain circumstances. When naturally colored and flavored (unoxidized) juices are desired, the decanter provides a useful alternative to presses because it is easily inert gas blanketed. Utilization of a metallic ultrafilter as a press has been patented but has not achieved commercial utilization (Beveridge & Rao, 1997).

1 kg pear of Dangshan pear, purchased from Hefei City Zhougudui fruit market selected, was pressed with adding 500 mL water to get the pear juice, and 10 g sodium erythorbate was added to protect the color. 2.5 g pectinase was added into the juice and then bathed at 50°C for 60 min in order to improve the juice yield, and followed by NaOH addition to adjusted at pH 6.5(Hu & Li, 1995).

Mature, ripe, healthy bananas (*Musa Sapientum*) were purchased from a local market in Obolo town, Nigeria. Each of the banana fruits was washed, hand peeled, and the edible portion (pulp) was sliced with a stainless steel knife. The slices were blended with 100°C hot water (1:4, pulp; water) in a Kenwood blender. The slurry was filtered through a double folded cheese cloth to obtain the juice. The juice was ameliorated to 18°Brix with sucrose and then 0.5 mg/100ml Potassium metabisulphite was added. The juice was then pasteurized (60°C, 3 m) and held in a freezer at 10 ± 2°C until required (Akubor et al, 2003).

The highest juice yields were obtained from a combined interaction of temperature, maceration, application of pectolytic enzyme, and previously frozen fruit. Freezing mayhaw fruit prior to juice extraction increased juice yield regardless of the method used in extraction. The average juice yield obtained from previously frozen fruit was 157% higher than when using fresh fruit. Maceration of fruit plus the application of heat to the fruit pulp prior to juice expression had a significant influence in juice extraction efficacy for both fresh and frozen fruit. When heat was applied to fruit pulp prior to expression, juice yields

increased from 31.4% for cold press extraction and 63.5% for hot press extraction. Application of a pectolytic enzyme to preheated fruit pulp increased total soluble solids (TSS) in extracted juice by 15% when utilizing frozen fruit compared to hot-press extraction without enzymes yield of 6.6%. There was no significant difference in TSS recovered by either fresh or frozen cold press methods without enzymes. All steam extraction methods utilizing fresh fruit had a lower percent recovery of total acid (TA) than when a pectolytic enzyme was added to fresh fruit before extraction. There were no significant differences in juice pH regardless of whether fresh or frozen fruit were used. Recovery of both TSS and fructose was significantly increased when pectolytic enzyme was added to fresh fruit. The lowest recovery of fructose was from steam extraction for both fresh and frozen (Trappey et al., 2008).

The juice extraction from fruits and vegetables can be done by size reduction methods as crushing, chopping and comminuting. This is usually employed for fruits that are fully ripened and too soft for whole or diced or sliced packs.

Crushing of products by chopping them into small pieces (0.025-0.075 inch) prior to heating speeds enzyme activity. Hence it is required to remove the air at the time of crushing and immediately after comminuting.

### **c. Pre-treatment**

Most of the studies concerning pear juice deal with the problem of the rapid browning during processing. Heat treatment, ascorbic acid, and SO<sub>2</sub> have been applied to prevent browning during processing. Thermal treatment, the only permanent treatment, and ascorbic acid have resulted in production of pear concentrates of lighter color (Petropakis & Montgomery 1984). Approximately 300 ppm of ascorbic acid was added to prevent browning in pear pulp and juice during the time of heating (Montgomery & Petropakis, 1980). Ascorbic acid, however, is not always desirable as it can participate in nonenzymatic browning in later storage (Tatum et al., 1969). Heating the fruit mash is an alternative. However, heating pears solubilizes middle lamella pectins resulting in cell separation and saucing of the fruit. Serious problems in extraction and clarification of juice from such mashes have been encountered (Beveridge, 1986).

The pretreatment of Banana must with (0.05% W/W AT 40°C for 2h) pectinase followed by treating with amylase (0.05% W/W at 50°C for 3h) enhance the hydrolysis of complex carbohydrate like pectin and starch which leads to increase in clarity, decrease in the viscosity (55%), 2.7 fold increase the amount of extracted juice a 15% and 39% increases in total soluble sugar and reducing sugar in extracted juice, respectively (Cheirsilp & umsakkul, 2008).

Pawpaw, Cashew, Banana and Pineapple wine were produced in the laboratory of the Department of Microbiology University of Ilorin, Ilorin, Nigeria using *Saccharomyces cerevisiae* purchased from E. C Kraus USA as a fermenting organism (Bisson & Butzkc, 2007). Ten Kilogram (10 kg) each of peeled, ripe, fresh and healthy fruits was blended with a sterilized blender to obtain the fruit pulp. The fruit pulp was mixed with warm water 45°C (in the ratio of 1:2) to obtain the 'must' needed for wine production. The must was sterilized with sodium metabisulphate solution to remove microbial contaminants. Standardized campden tablet, 30% sucrose and yeast nutrient were added to the must and allowed to stay for 24 hours, after which yeast was added (Berry, 2000).

#### Traditional thermal pasteurization

Thermal processing is the most widely used technology for pasteurization of fruit juices and beverages. Juice pasteurization is based on a 5-log reduction of the most resistant microorganisms of public health significance (USFDA 2001). The process could be accomplished by different time-temperature combinations.

#### Low temperature long time (LTLT)

Fruit juice has been traditionally pasteurized by batch heating at 63-65°C for relatively long time (D'Amico et al. 2006). This method has been replaced by high temperature short time treatment due to the undesirable quality changes during this process.

#### High temperature short time (HTST)

HTST treatment could minimize those undesirable quality changes made by batch heating due to the much less duration of heat treatment. Currently, HTST pasteurization is the most commonly used method for heat treatment of fruit juice. For example, orange juice is processed by HTST at 90 to 95°C for 15 to 30 s (Braddock 1999). And apple juice is treated by HTST at 77 to 88°C for 25 to 30 s (Moyer & Aitken 1980).

#### Non-traditional method

Thermal processing has been proven to be effective for preservation of fruit juice and beverages. However, thermal treatment tends to reduce the product quality and freshness. Nowadays, consumer demand for natural, healthy and convenient food products is fast growing, which leads to the innovation of novel food preservation technologies. Based on the literature, these novel technologies can be generally divided into physical methods (mostly non-thermal methods) and chemical approaches.

## Physical methods (Non-thermal pasteurization)

Some non-thermal pasteurization methods have been proposed during the last couple of decades, including high hydrostatic pressure (HHP), high pressure homogenization (HPH), pulsed electric field (PEF), and ultrasound (US). These emerging techniques seem to have the potential to provide “fresh-like” and safe fruit juices with prolonged shelf-life.

### High hydrostatic pressure (HHP)

High hydrostatic pressure (HHP) processing uses pressures up to 1000 MPa, with or without heat, to inactivate harmful microorganisms in food products (Ramaswamy et al., 2005). High hydrostatic pressure has traditionally been used in non-food areas such as ceramic and steel production. The application of HHP in food area started from 1900s when Hite and other researchers applied HHP on the preservation of milk, fruits and vegetables. In 1990, the first HHP processed fruit jams were sold in the Japanese market. Subsequently, HHP processed commercial products including fruit juices and beverages, vegetable products, among others, have been produced in North America, Europe, Australia, and Asia (Balasubramaniam et al., 2008).

HPP is proven to meet the FDA requirement of a 5-log reduction of microorganisms in fruit juices and beverages without sacrificing the sensory and nutritional attributes of fresh fruits (San Martín et al., 2002).

### Pulsed electric field (PEF)

Pulsed electric field processing (PEF) applies short bursts of high voltage electricity for microbial inactivation and causes no or minimum effect on food quality attributes. Briefly, the foods being treated by PEF are placed between two electrodes, usually at room temperature. The applied high voltage results in an electric field that causes microbial inactivation. The applied high voltage is usually in the order of 20-80 kV for microseconds. The common types of electrical field waveform applied include exponentially decaying and square wave (Knorr et al., 1994,).

Power ultrasound (US) has emerged as a potential non-thermal technique for preservation of food products over the last decade. Compared with diagnostic ultrasound, power US uses a lower frequency range of 20 to 100 kHz and a higher sound intensity of 10 to 1000 W/cm<sub>2</sub> (Baumann et al., 2005).

Ultraviolet light (UV-light) technology utilizes radiation with the electro-magnetic spectrum in the range of 100 to 400 nanometers, between visible light and x-rays. It could be further divided into UV-A (320–400 nm), UV-B (280–320 nm) and UV-C (200–280 nm). UV-C is

known to have biocidal effects and destroys microorganisms by degrading their cell walls and DNA. Therefore, UV-C could be used for the inactivation of microorganisms such as bacteria, yeasts, moulds, among others. The amount of cell damage depends on the type of medium, microorganisms and the applied UV dose (Ngadi et al., 2003). For fruit juice and beverage processing, the wavelength of 254 nm is widely used (Guerrero et al., 2004).

Ultrafiltration (UF) and microfiltration (MF) are the most commonly used membrane filtration techniques for clarification and pasteurization of fruit juice (Cassano et al., 2003). The effectiveness of the treatment depends on many parameters including processing factors such as types of membrane, pore size, transmembrane pressure and medium factors such as type of juice and microorganism. For example, an ultrafiltration (UF) unit, with polysulphone membranes of 10 kDa and 50 kDa pore sizes and trans-membrane pressures of up to 155 kPa, were used to treat apple juices. Results indicated that pH, acid content, and soluble solids did not change but presented less variability for the smaller pore membrane treatment. Relative colour changes were observed for both membranes, which was more detectable for the larger pore membrane treatment (Zarate-Rodriguez et al., 2001). Another application example was to use an ultrafiltration membrane of 15 kDa pore size to filter carrot and citrus juices. Then the clarified juices could be further processed by reverse osmosis and osmotic distillation (Cassano et al., 2003).

Chemical methods (natural antimicrobials)

Apart from physical methods, some chemical preservatives are widely used for the shelf-life extension of fruit juices and beverages. The most commonly used preservatives are potassium sorbate and sodium benzoate. However, consumer demand for natural origin, safe and environmental friendly food preservatives is increasing. Natural antimicrobials such as bacteriocins, lactoperoxidase, herb leaves and oils, spices, chitozan and organic acids have shown feasibility for use in some food products (Gould, 2001). Some of them have been considered as Generally Recognized As Safe (GRAS) additives in foods.

#### **d. Fermentation**

Fermentation is a relatively efficient, low-energy preservation process which increases the shelf life and decreases the need for refrigeration or other form of food preservation technology. Freezing of fruits and vegetables is not economically viable at the small scale. Fermentation requires very little sophisticated equipment, either to carry out the fermentation or for subsequent storage of the fermented product. It is a technique that has been employed for generations to preserve fruits in the form of drinks and other food for consumption at a later date and to improve food security. Basically, most fruits can be fermented if not all provided they are well prepared (Saranraj & Stella, 2012). It is, therefore,

a highly appropriate technique for use in developing countries and remote areas where access to sophisticated equipment is limited.

Fermented fruit wines are popular throughout the world, and in some regions, it makes a significant contribution to the diet of millions of individuals. Harvested fruits may undergo rapid deterioration if proper processing and storage facilities are not provided, especially in the humid tropics where the prevailing environmental conditions accelerate the process of decomposition. Although, there are several options for preserving fresh fruits, which may include drying, freezing, canning, and pickling, and many of these are inappropriate for the product and use on small scale in developing countries. For instance, the canning of fruits at the small-scale has serious food safety implications and contamination, especially botulism. (Sarah, 2017).

Ethanol fermentation performed by yeast and some types of bacteria break the pyruvate down into ethanol and carbon dioxide. It is an important thing in bread making, brewing, and winemaking. Usually, only one of the products is desired; in bread-making, the alcohol is baked out, and in alcohol production, the carbon dioxide is released into the atmosphere or used for carbonating the beverage. When the ferment has a high concentration of pectin, minute quantities of methanol can be produced (Meyer, 2017).

There are many fermented drinks made from fruit in Africa, Asia, and Latin America. These include drinks made from bananas, grapes, and other fruits. Grape wine is perhaps the most economically important fruit juice alcohol. It is of major economic importance in Chile, Argentina, South Africa, Georgia, Morocco, and Algeria. Due to the commercialization of the product for industry, the process has received most research attention and is documented in detail. Banana beer is probably the most widespread alcoholic fruit drink in Africa and is of cultural importance in certain areas. Alcoholic fruit drinks are made from many other fruits including dates in North Africa, pineapples in Latin America, and jack fruits in Asia (Steinkraus, 1996).

## **e. Clarification and Stabilization of Wine**

In winemaking, clarification and stabilization are the process by which insoluble matter suspended in the wine is removed before bottle. This matter may include dead yeast cells (lees), bacteria, tartrates, proteins, pectin's, various tannins and other phenolic compound, as well as pieces of grape skin, pulp, stem and gums.

Clarifying agents or fining is the addition of a reactive or adsorptive substance to remove or reduce the concentration of one or more undesirable constituents for the purpose of enhancing clarity, color, aroma and/ or stability modification (Bruce et al. 1995). The most commonly used fining agents perform their tasks by attracting the positively and negatively charged particular in the unclear wine, the fining agents, also have positive and negative

charger. Example includes gelation, Casein, albumin, Kaolin, bentonite (-), gelatin (+) sparkolloid (-), and egg white (+), and silicon dioxide or kieslsel (-). They work by adsorption: Condensing and holding a gas or soluble substance upon their surfaces. Bentonite is probably the choice of most winemaker, as it is very easy to prepare and use. It is very effective in dragging out yeast, tannins and other stubborn protein based particles that may want to linger after fermentation (Berry, 2000).

During the last years conventional clarification process has been supported by the use of ultrafiltration membranes. This has resulted in the production of clear and sparkling wine.

## **f. Maturation**

Wine ageing is an important process to produce high-quality wines. Traditionally, wines are aged in oak barrel ageing systems. However, due to the disadvantages of the traditional ageing technology such as lengthy time needed, high cost, etc., innovative ageing technologies have been developed. These technologies involve ageing wines using wood fragments, application of micro-oxygenation, ageing on lees or application of some physical methods. Moreover, wine bottling can be regarded as the second phase of wine ageing and is essential for most wines. Each technology can benefit the ageing process from different aspects. Traditional oak barrel ageing technology is the oldest and widely accepted technology. The application of wood fragments and physical methods are promising in accelerating ageing process artificially while application of micro-oxygenation and lees are reliable to improve wine quality. The impacts of operational parameters of each technology on wine quality during ageing are analyzed, and comparisons among these ageing technologies are made. In addition, several strategies to produce high-quality wines in a short ageing period are also proposed.

Occasionally the ageing process can be divided into the two phases. The first one is called maturation, which refers to change in wines after fermentation and before bottling, and the second phase of ageing is bottling. During wine ageing group of subtle reaction occur, which tend to improve the taste and flavor of wine over time. Traditional ageing technology is based on the storage of wine in oak barrels due to the positive effects on sensory characteristic and the enrichment of wood relates aromatic compounds in wine (Ortega et al., 2004).

During alcoholic fermentation large groups of aromatic compound are formed, including higher alcohols, aldehydes, esters, acid, ketones and other. Ageing can modify their compound and give wine their district fragrances. Woods also transfer a series of aromatic substances to wines when they are aged in oak barrels. The volatile compounds extracted from woods, are mainly furfural compounds, guaiacol, oak or whiskey lactone, eugenol, vanillin syringaldehydes and volatile phenols. (Zeng et al., 2008).

## **1. Barrel ageing system**

In the barrel ageing system, a small amount of oxygen can reach the inner barrel through the semi-permeable walls and many chemical reactions tend to occur near the interface of inner barrel wall. Therefore, a concentration gradient of the products builds near the barrel wall and the accumulation of these products may form a reduction barrier, which prevents fresh wines from reaching the neighborhood of barrel wall. Consequently, the ageing reactions are slowed. Recently, a non-deleterious barrel ageing system was developed to breach the reaction barrier and accelerate ageing (Eustis, 2010). In this semi-permeable wine ageing container, either an internal device or an external device can be fitted, such as an internal circulating pump and an external pump, so that a liquid motion is induced mechanically and fresh wine is brought to the neighborhood of barrel wall to accelerate ageing. Furthermore, Eustis (2010) claimed that with the help of the mechanical motion of liquid, the oxygen transfer from barrel surface to the inner barrel can increase.

### Disadvantages of Oak barrels

Ageing time, high price, cannot be used for long time, as barrels become older contaminated by miserable microorganism, yeast genera *Brefanonye* and *Dekkera*. These yeast can produce significant concentration of ethyl phenols which have unpleasant horsey and medical aromas; wine loss due to evaporation cause financial losses to winemakers (Adana et al., 2005).

Several strategies are proposed to produce high quality wines in a short ageing period, which may be an important research area in the future and benefit the modern winemaking industry.

## **2. Wine ageing using wood fragments**

One of the alternative ageing systems involves wood fragments addition, such as oak chips and oak staves into wines. Up to now, the addition of wood chips to wines has been applied in the last decades to provide them an oak flavour in Australia, the USA, South Africa and South America. Since October 2006, the European Union also approved the use of pieces of oak wood during winemaking, but wines treated in this way should be labeled (Bautista et al., 2008). However, it should be taken into account that the alternative ageing system in the presence of wood fragments cannot replace the traditional ageing system completely, especially for long-term ageing. For example, in the study of the evolution of aromatic compounds in Monastrell red wines aged both in oak chip systems and American oak barrels, it was found that chips released aromatic compounds into wine rapidly in the first three months of ageing, and significant quantities of cis- and trans-oak lactones and vanillin were detected (Bautista et al., 2008). However, in the following 6 months, the concentrations of these compounds remained constant or decreased while the wines aged

in new and used barrels continued to extract aromatic compounds for a long time. The overall quality of wine aged in new barrels was also better than that aged with chips.

The differences in basic enological parameters and phenolic compositions between wines treated with wood fragments and those aged in barrels could augment as the length of wood contact time increases (Álamo et al., 2008). From an organoleptic point of view, the application of wood fragments can be regarded as a good alternative to barrels for producing short-term aged wines with satisfied quality. However, in some cases the sensory quality of wines aged with wood fragments cannot be as good as those long-term aged wines in new barrels. On the other hand, during the phase of bottling, barrel-aged wines also behave differently from those aged with wood fragments. In this sense, a quicker loss of anthocyanins was found during the bottling of red wines treated with wood fragments than those aged in barrels (Sanza & Domínguez, 2006).

Similar to oak barrels, toasting is a conventional pretreatment of wood fragments. It can increase the amounts of aromatic compounds, including furfural, 5-methylfurfural, eugenol, vanillin, guaiacol and its derivatives in wood (Sarni et al., 1990).

### **2.1 Wood fragment toasting and its significance**

Different toasting pretreatments of wood fragments can affect the final wine quality and its influences on wine chemical compositions and sensory characteristics are even greater than the type of wood used (Guchu et al., 2006).

The toasting level of wood fragment is related to the production of wood-related volatile compounds and Bozalongo et al. (2007) pointed out that medium and heavy toasting can produce more contents of 5-hydroxymethylfurfural, 5-methylfurfural, furfural, vanillin, 4-methylguaiacol, guaiacol and syringol than light toasting in wood fragments. During wine ageing, wines treated with medium toasted oak chips extracted the highest contents of furfural and cis oak lactone followed by those aged with heavily and lightly toasted chips in the study of Koussissi et al., (2009). Moreover, the effect of toasting level of wood fragments on sensorial characters of wine is also significant. Koussissi et al. (2009) found that heavy toasting gave wines higher wood-related properties, but also made wines more astringent and bitter. Therefore, through controlling toasting level of wood fragments, desired sensory characters of wine can be obtained.

Another factor which should be taken into account during wood fragment selection is the botanical characters and their geographical provenances. As discussed above, there are differences in volatile composition between woods of different species or origins. Besides the size and pretreatments of wood fragments, the contents of aromatic compounds extracted by wines also depend on the species of wood and their origins. For example, the Chardonnay wine treated with Hungarian oak chips (*Quercus petraea*) extracted trace

quantities of oak lactones whereas the same wine treated with American oak chips (*Quercus Alba*) extracted significant quantities during the 25-day ageing period (Guchu et al., 2006).

Moreover, a red wine macerated with Spanish chips (*Quercus pyrenaica* and *Quercus petraea*) was richer in furanic aldehydes and eugenol whereas those macerated with American chips (*Quercus Alba*) contained higher concentrations of cis-whiskey-lactone, vanillin and methyl vanillate (Rodríguez et al., 2009).

Therefore, wood fragment should be selected with extreme care by considering the whole characteristics of wood. In the presence of wood fragments, the ageing time can be reduced due to the high extraction rate of aromatic compounds. However, colour evolution commonly requires a longer maturation time and a small amount of oxygen to promote the chemical reactions.

### **3. Combination of micro-oxygenation with wood fragments during wine ageing**

This technique is based on the introduction of a small amount of pure oxygen into wines over time. At the beginning, the micro-oxygenation process was applied to imitate the barrel ageing during the 1990s. Nowadays, the main purposes of micro-oxygenation are to develop colour stabilization, strengthen red colour in red wine, enhance health of yeasts during alcoholic fermentation, improve the taste and structure of wine, stimulate oak barrel ageing system, modify aromatic characters of wine and remove undesired flavors (Gómez & Cano, 2010).

Oxygen dosing is a key factor for micro-oxygenation treatment. An excessive oxygen dosage can be very harmful to wine. To better manage the micro-oxygenation process, several variables, including free SO<sub>2</sub>, ethanol, colour indicators, phenolic compounds and dissolved oxygen available in wine should be monitored (Nevares et al., 2010). The changes of these variables are related to the micro-oxygenation treatment and can reflect the influence of this treatment on wine.

The characteristics of wood fragments play an important role in determining the oxygen consumption by wine because, on one hand, micro-oxygenation treatment is usually accompanied with addition of wood fragments and, on the other hand, oxygen consumption is closely related to the compounds released from wood to wine and the evolution of these compounds during ageing (Álamo et al., 2010). First of all, the evolution of oxygen consumption in wine depends on the toasting levels of wood fragments.

### **4. Wine ageing on lees**

Traditionally, lees are used during the ageing of natural sparkling wines, white wines and biological aged sherry wines produced in the presence of flor yeasts. Currently, this technique has been applied in all viticultural areas since it provides high-quality products.

From a sensorial point, ageing on lees can not only reduce bitterness and astringency, enhance the structure, roundness and body of wines, but also make the aromatic notes of wines more complex and persistent (Barrio et al., 2011). Generally, lees are mainly made up of yeasts, and in minor proportion, of some inorganic compounds and tartaric acid. This ageing option is coupled with either barrel ageing or ageing carried out in other containers, such as stainless tanks and large cooperage systems.

During ageing on lees, lees undergo a self-degradation process known as autolysis (Leroy et al., 1990). The cell wall is gradually degraded over time and several compounds, including polysaccharides, amino acids, peptides, fatty acids and lipids are released. Therefore, the composition of wine is affected by yeast lees. Firstly, the presence of lees during ageing can modify the aromatic properties of wines. On one hand, the contact of wines with lees cell walls can decrease the contents of some volatile compounds due to the sorption phenomenon (Pozo et al., 2009). It has been proved that lees are effective in removing 4-ethylphenol and 4-ethylguaiacol, which can reduce the quality of wine at high concentrations (Chassagne et al., 2005). Besides undesired volatiles, lees can also bind oak-related compounds and attenuate the impact of wood on wines, with the most affinity components being 4-propylguaiacol, 4-methylguaiacol, eugenol, furfural and 5-methylfurfural (Moreno & Azpilicueta, 2007).

For example, Liberatore et al. (2010) found that lees enriched flavour active compounds, like lactones, ketones, terpenoids, esters and aldehydes in Chardonnay white wines during ageing. Besides these mentioned effects of lees on aromatic composition of wines, lees may bring some risks due to the production of some sulfur odours and bad volatile compounds (Palacios et al., 1997). To reduce the generation of sulfur off-odours and avoid the evolution of low redox potential in lees, periodic stirring is employed during ageing on lees.

## **5. Development of Wine Ageing in Bottles**

Apart from ageing in barrels and other vessels, bottling is another important stage for wine ageing. Sometimes wines are directly aged in bottles (Segade et al., 2009). During storage in bottles, temperature, illumination, position and oxygen content are important to determine the composition of final products. Storage temperature should be considered carefully during bottling. Generally, wine cellar temperature is between 13°C to 15°C, and the storage temperature in the range from 5°C to 18°C is also acceptable as long as it keeps constant. Winemakers can store wines at low and controlled temperature whereas wines commonly stay at room temperature after they are put in sale places or purchased by consumers. Therefore, it is necessary to clarify how wine quality evolves at different temperatures during bottling. Zalema & Colombard white wines stored at a low temperature (4°C in the refrigerator) could be clearly distinguished from those stored at the ambient temperature and those stored at a constant temperature from 15°C to 20°C according to the linear

discriminate analysis of colour parameters and phenolic and volatile compounds (Hernanz et al., 2009). Furthermore, Garde-Cerdán et al. (2008) pointed out that low temperature (5°C) could enhance the contents of some important aromatic compounds such as ethyl esters of fatty acids and isoamyl acetate during the storage of a white wine without the addition of SO<sub>2</sub>. Blake et al. (2010) also confirmed that a relative low temperature (12°C-storage) benefited the quality of final wines compared to a 22°C storage condition since the lower temperature bottled wines showed higher retention ability of acetate esters, phenolic compounds (in red wines), free and bound SO<sub>2</sub>, and a lower browning index. In some cases, high temperature is useful for accelerating the ageing. Nevertheless, high temperatures should be avoided during wine ageing process in most cases, since they can sharply reduce the contents of aromatic compounds in wine and accelerate the process of browning of white wine (Berg & Akiyoshi, 1956).

## **6. Acceleration of Wine Ageing with Physical Methods**

Besides the above-mentioned technologies, there are several physical methods showing great potential for accelerating the wine ageing process, involving ultrasonic waves, gamma rays, electric fields and nanogold photocatalysis.

### **2.4 Use of herbs or medicinal plants in wine**

The technique of winemaking is known since the dawn of civilization and has followed human and agricultural progress. The earliest biomolecular archaeological evidence for plant additives in fermented beverages dates from the early Neolithic period in China and the Middle East when the first plants and animals were domesticated and provided the basis for a complex society and permanent settlements (McGovern & Zhang, 2004). In ancient China, fermented beverages were routinely produced from rice, millet, and fruits (Mena, 2012). However, in earlier years in Egypt, a range of natural products, specifically herbs and tree resins, were served with grape wine to prepare herbal medicinal wines. Many of the polyphenols and other bioactive compounds in the source materials are bonded to insoluble plant compounds. The winemaking process releases many of these bioactive components into aqueous ethanolic solution, thus making them more biologically available for absorption during consumption (Shahidi, 2009).

### **2.5 Analytical techniques for characterizing foodstuffs**

A wide variety of analytical techniques have been standardized for characterizing various foodstuffs mainly wine, honey, tea, olive oil, and juices. Simultaneously, consumer preferences for wine selection depend on several properties such as pleasant color, taste, aroma, ecological production, guaranteed origin, quality, and sensory perceptions offered by the complex combinations of hundreds of components present in wine (Saurina, 2010).

No food or beverage is worth producing, distributing, or marketing without having an approximate idea that its sensory quality is accepted by consumers (Tuorila & Monteleone, 2009). Apart from grapes, there are many other fruits available that can be used as substrates for winemaking. Among various fruits, grapes are the most technically and commercially used as substrates for winemaking. The impact of the model plant grape is relevant, and hence, genetic and molecular studies on this plant species have been proved to be very successful in winemaking (Pretorius, 2005). According to the routine definition, wine is a fermented beverage produced from grapes only. Otherwise, wine is given the prefix of the fruit from which it originates. Today, a big variety of fruits which differ in shape, color, taste, and nutritive value are available in the market and many are utilized widely for the production of fermented beverages.

## **2.6 Classification of Wine**

Wines come in various colors (red, white, and rose) and many types, which include dry and sweet, still and sparkling, and wines fortified with grape spirit (brandy) (Saranraj et al., 2013).

When dealing with fruits other than grapes, sugar may need to be added to spur the fermentation process in the event that the fruit does not contain enough natural sugar to ferment on its own in the presence of yeast. Some fruits such as cherries, raspberries, strawberries, and pineapples are also very high in acid, which can translate into a very sour tasting wine. In these cases, sucrose and water can be added to help counter the fruit's tart acidity (Karthikeyan, 2015).

Depending upon product manufacturing all wines can be classified as grape wine, fruit wine, berry wine, vegetable wine, plant wine, raisin wine etc. Grape wine is made exclusively from grapes and during the production process prohibited from using any other materials (exception is made only for sugar and oak barrels). Fruit wines are fermented alcoholic beverages made from a variety of base ingredients other than grapes; they may also have additional flavors taken from fruits, flowers, and herbs. These types of wines are made from pear, apple, banana, papaya, mango, jackfruit juice etc. Cherry wine is produced from cherries, usually those cherries that provide sufficient acidity to wine. Plant wine is produced from juice of trees like maple, birch, melons, watermelons, and other garden plants such as rhubarb, parsnips and rose petals. Raisin wine is made from dried grapes (raisins). Multiport wine is produced by mixing different kinds of grapes and wine materials.

Depending on the time of fermentation grape varieties and color fruit wines classified as are in red, white and pink wines.

## **1. White grape wine**

White grape wine is an alcoholic fruit drink between 10% and 14% alcoholic strength. This prepared from the fruit of the grape plant and is pale yellow in color (Ranken et al., 1997). There are many varieties used including Airen, Chardonnay, Palomino, Sauvignon Blanc, and Ugni Blanc. The main difference between red and white wines is the early removal of grape skins in white wine production. The distinctive flavor of grape wine originates from the grapes as raw material and subsequent processing operations. The grapes contribute trace elements of many volatile substances which give the final product the distinctive fruity character.

In the case of cashew, the apples are cut into slices to ensure a rapid rate of juice extraction when crushed in a juice press. The fruit juice is sterilized in stainless steel pans at a temperature of 85°C to eliminate wild yeast (Wilmasiri, 1971). The juice is filtered and treated with either sodium or potassium metabisulfite to destroy or inhibit the growth of any undesirable types of microorganism's acetic acid bacteria, wild yeasts, and molds. Wine yeast (*S. cerevisiae* var *ellipsoideus*) was added. Once the yeast was added, the contents are stirred well and allowed to ferment for about 2 weeks (Fundira, 2012).

After fermentation was completed, the wine is separated from the sediment by racking. It can also be clarified further using fining agents such as gelatin, pectin, or casein which are mixed with the wine. Filtration can be carried out with filter aids such as fuller's earth after racking. The wine was then pasteurized at 50°C–60°C. The temperature should be controlled, so as not to heat it to about 70°C, since its alcohol content would vaporize at a temperature of 75°C–78°C (Common wealth scientific and industrial research organization, 2013). It is then stored in wooden vats and subjected to aging. At least 6 months should be allowed for aging. If necessary, wine is again clarified before bottling. During aging and subsequent maturing in bottles, many reactions, including oxidation, occur with the formation of traces of esters and aldehydes, which together with the tannin and acids already present to enhance the taste, aroma, and preservative properties of the wine (Van & Tromp, 1982).

## **2. Red wine**

Red wine is made from red grapes, which are actually closer to black in color. There are many different types of red wines. This is considered to be the most classic in the kingdom of wines, mixing the delicious red grapes with a wide range of aromas, from oak to eucalypti, chocolate or even mint hints. The juice from most black grapes is greenish-white; the red colour comes from anthocyanin pigments present in the skin of the grape.

### **3. Pink wine**

Pink wine having a light pink color, grape skin removed immediately after the start of the fermentation process. These wines are made from a mixture of "black" and "white" grapes, using the technology of producing white wines (Baslingappa et al., 2016).

### **4. Fortified wines**

The liqueur wines are those sweet wines obtained from so-called "base products" (grape must in fermentation; wine; a combination of both products; mixture of wine and must; or grape must or its mixture with wine) to which has been added neutral alcohol of vine origin or dried grape distillate and, in some cases, sweeteners products from grapes such as concentrated grape must.

Their preparation necessarily requires the addition of neutral grape alcohol, to the wine during fermentation, what stops the fermentation process, and kills the yeast that consumes the sugar, in order to achieve an alcoholic content and residual sugar level according to the characteristics of each type of wine. The law states that actual alcoholic strength for these wines does not be less than 15 % vol. and a total alcoholic strength does not be less than 17.5 % vol. (Council Regulation 479/2008), except for certain liqueur wines with a designation of origin or with a geographical indication which have their own regulations (Patricia et al., 2013).

### **5. Sparkling grape wine**

Sparkling grape wines are made in the Republic of South Africa. Sparkling wines can be made in one of three ways. The cheapest method is to carbonate wines under pressure. Unfortunately, the sparkle of these wines quickly disappears, and the product was considered inferior to the sparkling wines produced by the traditional method of secondary fermentation. This involves adding a special strain of wine yeast (*S. cerevisiae* var *ellipsoideus*) - champagne yeast to wine that has been artificially sweetened. Carbon dioxide produced by fermentation of the added sugar gives the wine its sparkle. In the original champagne method, which is still widely used today, this secondary fermentation is carried out in strong bottles, capable of withstanding pressure, but early in the 19th century, a method of fermenting the wine in closed tanks was devised, this being considerably cheaper than using bottles (Mohanty, 2006).

## **2.7 Sensory analysis**

Tasting wine has always been part of winery operations. Oenologists and winemakers have long used their senses to assess wine quality at every stage of production. These professionals are often referred to as experts; they tend to rely on their own holistic

assessment, the result of objective and subjective considerations and tasting experience. Wine evaluation rests on both sensory and cognitive mechanisms. Sensory mechanisms are physiologically based, pertaining not only to taste and smell but also to the visual and tactile senses. Cognitive mechanisms are experience based, including wine-related knowledge, memory, expectations, and the context in which evaluation occurs. Sensory mechanism entails “bottom up” processes, reflecting the influence of the sense organs. Cognitive mechanisms entail “top-down” processes, reflecting knowledge, expectations, beliefs, desires, and motives.

The main advantage of sensory techniques over traditional wine tasting is the ability to collect wine assessments in the least biased way. This is accomplished by using protocols minimizing physiological and psychological factors known to affect human sensory responses and utilizing assessors which are highly sensitive to sensory stimuli and able to evaluate their perception analytically and objectively. Sensory professionals often make the analogy between a sensory panel and a high-tech analytical instrument, similar to what a gas chromatography– mass spectroscopy (GC–MS) could represent for flavour chemists. Analytical data collected from sensory panels are expected to be accurate, sensitive, repeatable and reproducible. Another branch of sensory science includes hedonic tests, designed to determine consumer preference between two or more products, and also requiring ad hoc protocols minimizing any factors that could influence consumer appreciation. Sensory techniques have been used mainly to support research efforts in viticulture and oenology.

### **2.7.1 Tasting environment and best practices**

Sensory evaluation is a scientific discipline used to evoke measure, analyze and interpret reactions to stimuli perceived through the senses (ASTM, 2000). Similar to wine chemical analysis (e.g. alcohol, titratable acidity), sensory tests must be conducted under standardized and controlled conditions minimizing psychological and physiological biases affecting human responses. Implementation of good sensory practices is therefore critical in maintaining the integrity of the sensory test and ensuring the objectivity of the results.

#### **1. Good sensory evaluation practices**

It is important to remove all marketing cues that can alter perception of quality. Samples are always presented uniformly, in identical containers coded with random numbers to prevent bias from extraneous clues such as brand or treatment. Standard tulip-shaped clear wine glasses are optimal for evaluation of aroma, while plastic cups or beakers can be used when only taste or mouth feel are being evaluated. Providing watch glasses or Petri dishes as sample lids during aroma evaluations increases aroma intensity and reduces odours in

the tasting facility. For evaluation of flavour, differences in appearance are normally masked by serving samples in opaque containers (e.g. black ISO glasses) or under red light. Analytical sensory tests must be conducted in facilities that prevent any visual, audio or olfactory distractions. For example, the company break room is not a good location since reminiscent odours of coffee or food could disturb wine evaluation. The environment in which sensory tests are conducted should be temperature- controlled, quiet and odour-free. Positive pressure inside the tasting room is recommended although not mandatory if the ambient air can be quickly regenerated through air cleaners or by opening windows (assuming the outdoor air is not contaminated). Hedonic tests are typically conducted in a central location. Central location settings should simulate a natural context of consumption as much as possible, as recent research showed that liking scores can vary substantially between a laboratory (clinical) environment and a real restaurant (Petit & Sieffermann, 2007). Whether their participation involves simple or complex tasks, panelists should not be the experimenters (who administer the tests). In cases in which panellists are required to pour or distribute their own samples, error can be introduced by non- uniform sample sizes and by mix-up of samples. Further, panellists may learn or speculate about the design of the experiment. Asking panellists to perform complex tasks on their own, such as initiating timing with a stopwatch, tasting a sample and turning a device on and off at specified times, distracts from their ability to concentrate on rating intensity and introduces error. Instructions given to panellists to perform the test must be clear and non- ambiguous. Experimenters should not assume written instructions are meaningful by themselves; moreover, experienced panellists tend to partially read instructions, especially if a task looks familiar, although the procedure might have changed. Studies must be designed to account for sequence effects in tasting wines that influence human responses. In all analytical sensory tests, samples are usually expectorated rather than being swallowed to reduce any change in response due to satiety, fatigue or, in the case of alcoholic beverages, increase in blood alcohol. For affective tests, consumers are instructed to consume wine as they would normally do, which entails swallowing the product. First position and carry-over effects may alter sensory perceptions when evaluating wine. For example, astringency and bitterness are perceptions that can build up over repeated ingestions, leading to over-estimated intensity rating of these attributes (Noble, 2002). Use of a pectin solution (followed by thorough rinses with water) helps reduce bitterness or astringency of red wine (Colonna et al., 2004); water rinsing and eating unsalted crackers or soft bread crumbs are also efficient strategies. Other techniques to reduce sensory fatigue and remove impact of previously tasted or smelled samples include breathing fresh air, sniffing or rinsing with water between samples or resting between samples. The specific inter- stimulus protocol varies with each product.

## **2. Panel**

An analytical sensory panel is generally formed of eight to 20 individuals, selected from within the employee pool if the company is large enough (internal sensory panel) or from the community (external panel). Both panel types have advantages and disadvantages and the final choice will depend on the resources available in the company and the type and the volume of sensory tests needed to guide the organization's activities. To become a member of a sensory panel, candidates are screened according to their sensory acuity, availability and motivation (Meilgaard et al., 2007). Further, selected candidates require training to perform sensory tasks objectively and consistently, unlike instruments, although both need to be calibrated and tested for reproducibility. The objectives of training are to familiarize panellists with the tasting methods and the products under study. For novice panellists, a generic training is recommended to teach them the basis of sensory physiology and psychology, good sensory practices in the booth, the data collection system and, finally, to enlarge their sensory knowledge by evaluating a large array of products exhibiting different sensory properties. Wine professionals working in the organization should be considered for panel membership. They have usually been trained in wine appreciation (e.g. through the Wine and Spirit Education Guild) or in sensory evaluation taught at a university or college. However, this sensory training should not excuse them from recalibrating their knowledge and sensory references prior to a study. Moreover, their wine expertise could mislead them, as shown in several experiments where experts evaluated wine using cognitive strategies rather than responding to their actual sensory perceptions (Castriota et al., 2005).

Conducting hedonic tests requires identification of typical consumers of the product category for recruitment. Winery personnel or 'experts' should not be used unless they represent the typical consumer. Therefore, it is critical to define the targeted consumers to be recruited in terms of demographics, purchase and consumption habits based on the existing knowledge of the typical consumers of a product category, or based on the consumer targeted to become users of the new product. Because of the tremendous variation in preferences, a large number of target consumers ( $n > 50$ ) must be recruited, especially when conducting quantitative tests. Geographical and cross-cultural differences may create different responses from consumers in product liking; therefore these factors need to be considered in the screening of potential respondents and in the selection of the location of the test.

# CHAPTER THREE

## MATERIALS AND METHODS

### 3.1 MATERIALS

#### 3.1.1 Equipment

Equipment provided by the Central Department of Biotechnology, Kritipur, such as hot air oven (Acumen Equipment India Pvt Ltd), Orbital shaking incubator (Ascent India), spectrophotometer (GENESYS 10VIS), refract meter, distillation apparatus, gas chromatography and alcohol meter were used for the research.

#### 3.1.2 Chemicals used

Methanol, Folin-ciocalteau reagent, and ethanol manufactured by Thermo-Fisher Scientific India Pvt Ltd, potassium metabisulphite, sodium carbonate, sodium hydroxide, sodium thiosulphate, sodium bisulphate, sodium bicarbonate manufactured by Merck specialities Pvt Ltd, Gallic acid manufactured by Molychem were used in the research. Media such as YEPD agar, YGP broth, PDA agar, starch agar, glucose, yeast extract manufactured by HIMEDIA Laboratories Pvt Ltd were used for screening and isolation purpose.

#### 3.1.3 Sampling of *murcha*, pear and other fruits

*Murcha* were collected from places such as Lubhu, Palpa, Jorpati and Sunsari whereas fruits like grapes, raisins, apricot, and apples were purchased from the local market and flower *Argyrea nervosa* were collected from within the Tribhuvan University. Ripened pears fruit were collected from Pharping, Kritipur. A 60- 80 kgs of firm, crispy and juicy healthy pears were brought from Pharping. The *murcha*, fruits and flower were collected in sterile plastic bags. The samples were transferred to the Lab at Central Department of Biotechnology, Kritipur and stored in refrigerator at 4°C for further. The pears were kept at room temperature.

#### 3.1.4 Media used

YEPD agar was used for isolation and subculture of yeast. YEPDA is the media that can grow fungus and avoid growth of any bacteria by using chloramphenicol (0.05g/L). And yeasts are the only group of fungus that is unicellular and round or oval shaped growing in a colony. The composition of media is presented in appendix.

## **3.2 METHODOLOGY**

### **3.2.1 Microbiological analysis**

#### **1. Total yeast count and wine yeast enumeration**

The refrigerated murcha, fruits and flower samples were subjected to the procedure for isolation. Pour plate technique was used to isolate the organisms. Murcha samples were directly diluted to  $10^{-1}$ ,  $10^{-2}$ , up to  $10^{-10}$  using sterile water. Samples such as apples, raisin, apricot, grapes and flower were cut in 5mm cubes and directly placed onto the petriplate containing a firm YEPD agar media. The selected colonies were purified with streak plate technique. The isolates were examined according to their colony morphologies and Simple staining using standard protocol.

In simple staining, the yeast smear is stained with a single reagent. The most commonly used basic stains are methylene blue, crystal violet and carbon fuchsin. Since all the fungi are gram positive hence, simple staining is done for the study of morphological characters.

#### **2. Selection of potential wine yeasts**

For the selection of wine yeasts among the diverse colony of yeast obtained during enumeration, the major selection criteria chosen were ability to grow at different sugar concentration,  $SO_2$  tolerance test, alcohol tolerance test and alcohol determination test.

##### **a. Ability to grow at different sugar concentration**

According to Hui et al., (1994) sugars are the most common substrate of fermentation to produce ethanol, lactic acid, and carbon dioxide. Although sugar is an important substrate of fermentation, higher sugar concentration inhibits the growth of microorganisms. At higher concentrations more than 40%, only a certain group of yeasts - the Osmophilic type - can survive. There are only a few yeasts that can tolerate sugar concentrations of 65–70% and these grow very slowly in these conditions (Board, 1983). A winemaker who wishes to make a wine with high levels of residual sugar (like a dessert wine) may stop fermentation early either by dropping the temperature of the must to stun the yeast or by adding a high level of alcohol (like brandy) to the must to kill off the yeast and create a fortified wine (Robinson J, 1983). In order to screen the yeast strains for their tolerance to a high concentration of glucose, one loopful of selected colony was inoculated in YPD broth containing 20%, 25%, and 30% dextrose at 28°C for 24 hours and observed for the viable colonies on YPD agar, on 48 hours of incubation at 28°C.

### **b. Sulphur dioxide tolerance test**

Although SO<sub>2</sub> is highly toxic to bacteria, Native yeasts and molds, commercial wine yeast strains are selected to have a higher tolerance to SO<sub>2</sub>. It is well-known that minimal sulphiting (20 to 30 mg/L) before fermentation initially slows fermentation, but eventually fermentation is completed more rapidly. This phenomenon can be attributed to the killing or inhibition of native yeast strains, bacteria and moulds, which enhances the growth conditions for the yeast starter culture (Ribéreau et al., 1998). 20ml of YPD broth containing 20% dextrose and sulphur dioxide of 75ppm concentration was inoculated with loopful of colony and incubated for 24 hours. Then the OD of different colony was compared at 600 nm.

### **c. Alcohol tolerance test**

Ethanol tolerance has also been defined as the amount of ethanol resulting from sugar fermentation. In addition, it has been proposed that inhibition of fermentation is the best indicator of ethanol tolerance. Ethanol has three major effects on yeast cells. It inhibits cell growth, cell viability, and fermentation, although to different extents. Thus, the definition of ethanol tolerance would depend on which parameter one is considering. The most widely employed method for determining ethanol tolerance involves the suppression of cell growth in the presence of exogenous ethanol. Thus the strains were examined for their tolerance to high alcohol content by culturing them in YPD broth containing 10-18% alcohol under 28°C for 24 hour and then comparing the growth at OD 600 nm.

### **d. Study of changes in Total soluble solids**

Ethanol and CO<sub>2</sub> are the major end-products of fermentation. The rate and efficiency of ethanol production are important factors in winemaking fermentations. The production of ethanol is influenced by yeast strain, temperature, and growth medium. Yeast strains were cultured in YPD broth contains initially 20°Brix at 28°C. By using the hand refract meter which generally measure the TSS or degree brix gives the tentative measurement of alcohol. Changes in °Brix helps in calculate the alcohol % during fermentation process.

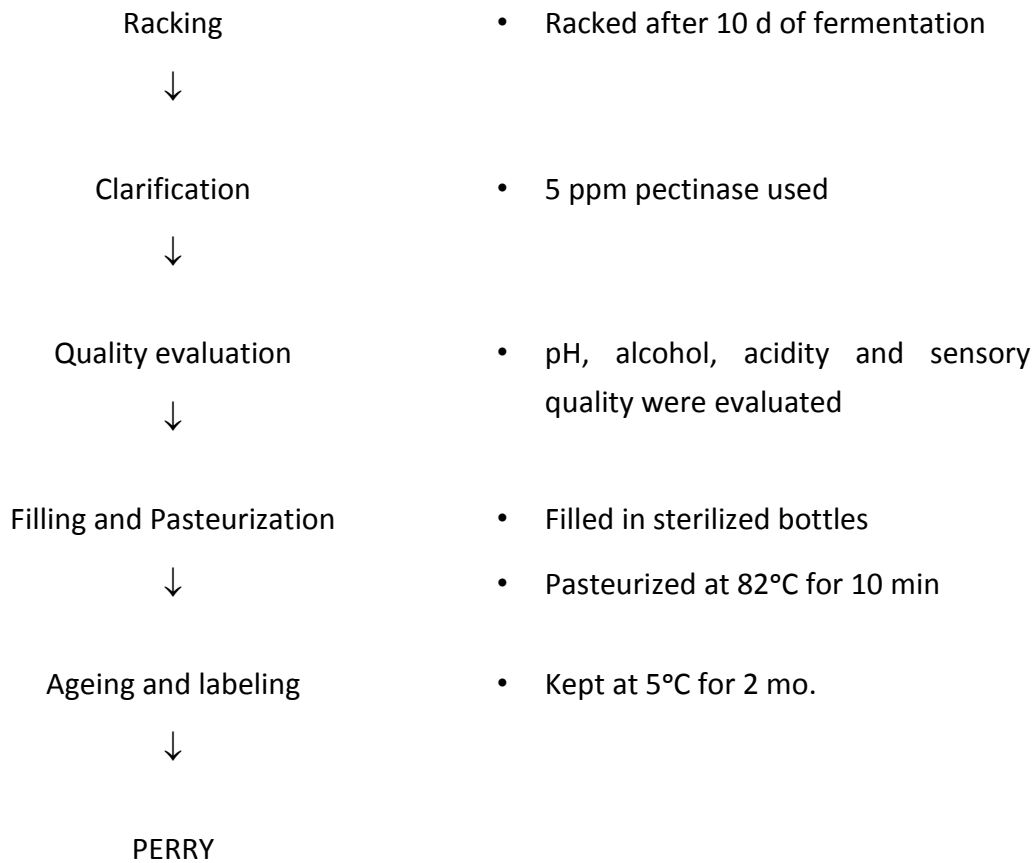
The colonies that gave the best or produced efficient results in the above four criteria were selected and the isolates were sub cultured in YPD agar plates for further research in the fermentation of pear juice.

### **3.2.2 Preparation of Perry wine**

Selected potential wine yeasts isolates from murcha (YPM) and flower (YFW) were used for the preparation of Perry wine. Commercial yeast (*Saccharomyces Bayanus* SN9) was also

used to prepare Perry wine in the same environment and condition for comparative analysis of the product.

Process	Notes
Pear	<ul style="list-style-type: none"> <li>Collected from Pharping, Kritipur</li> </ul>
↓	
Washing, peeling, cutting	
↓	
Juice extraction	<ul style="list-style-type: none"> <li>Manual juice extractor used</li> </ul>
↓	
°Brix adjustment	<ul style="list-style-type: none"> <li>Sugar was used</li> <li>Brix refractometer was used</li> </ul>
↓	
Pasteurization	<ul style="list-style-type: none"> <li>Done at 65°C for 5 min</li> </ul>
↓	
Cooling	<ul style="list-style-type: none"> <li>Cooled to 25°C</li> </ul>
↓	
Pitching	<ul style="list-style-type: none"> <li>3 loopful of pear culture used</li> <li>640 ppm yeast nutrient used</li> </ul>
↓	
Primary fermentation	<ul style="list-style-type: none"> <li>Done at Room temp. (12-26°C) for 14 days</li> </ul>
↓	
Racking	<ul style="list-style-type: none"> <li>Clear supernatant was racked into another vessel</li> </ul>
↓	
Secondary fermentation	<ul style="list-style-type: none"> <li>Glass jar was fitted with airlock</li> </ul>
↓	



**Fig. 3.1:** Process involved in the laboratory preparation of perry wine

### **a. Clarification of wine**

In winemaking, clarification and stabilization are the process by which insoluble matter suspended in the wine is removed before bottle. This matter may include dead yeast cells (lees), bacteria, tartrates, proteins, pectin's, various tannins and other phenolic compound, as well as pieces of grape skin, pulp, stem and gums. The clarificant example includes gelation, Casein, albumin, Kaolin, bentonite (-), gelatin (+) sparkolloid (-), and egg white (+), and silicon dioxide or kieslsel (-). They work by adsorption: Condensing and holding a gas or soluble substance upon their surfaces. Bentonite is probably the choice of most winemaker, as it is very easy to prepare and use. It is very effective in dragging out yeast, tannins and other stubborn protein based particles that may want to linger after fermentation (Berry, 2000).

Different clarifying agents employed to clarify Perry wine are as follows:

#### **a. Bentonite**

To determine the impact of bentonite on cloudy wine, for 20 litre of wine 12gm of bentonite is required since we had three jars containing 5L each. Hot water or wine sample about 20 ml was taken and mixed with 3gm Bentonite by calculation and allowed to stand for 4 hours

with continuous stirring at regular interval of one hour. Bentonite solution so prepared was mixed with 600ml of wine and finally the above 600ml bentonite mixed wine was added to the bottle containing 5 liter wine. The mixture was allowed the stand for two days to observe its impact on cloudy wine.

b. Dry gelatin

To determine whether gelatin clarify the cloudy wine 10ppm, 20ppm, 30ppm, 40ppm of gelatin solution was prepared by hydration with warm water at 45 degree. So prepared solution were transferred in 50ml wine contained in five falcon tube followed by mixing. The mixture was allowed the stand for two days to observe its impact on cloudy wine.

c. Egg white

To determine whether egg white clarify the cloudy wine, 6gm egg white was dissolved in 12gm 10% salt solution. From so prepared solution 300ul, 600ul, 900ul, 1200ul and 1500ul were drawn and transferred in 50ml wine contained in five falcon tube followed by mixing. The mixture was allowed the stand for two days to observe its impact on cloudy wine.

d. Pectinase

5mg, 10mg, 15 mg, 20 mg of pectinase solution was prepared by dissolving in sterile water and added in 50 ml of wine contained in five falcon tube. The mixture was allowed to stand for two days to observe its impact on cloudy wine.

### **3.2.3 Determination of pH, acidity and total soluble solids**

pH, titrable acidity and TSS were analyzed on day 1, 2, 3, 5, 7, 9, 11, 13, 15, 16 during the course of fermentation.

#### **1. Titrable acidity and pH determination**

pH was measured by calibrated pH meter. Acidity was measured by titrating the wine against 0.1 M NaOH, using Phenolphthalein as an indicator.

#### **2. Quantification of total solid content**

A hand-held refractometer can be used to measure % TSS (degrees Brix) of pear. Temperature affects the TSS reading. Increase about 0.5% TSS for every 5 °C increasing temperature. Standardize the refractometer between each reading with distilled water and reading should be 0% at 20 °C.

### **3.2.4 Organoleptic evaluation**

A panel of 6 untrained judges consisting of 1 female and 5 male were taken for sensory evaluation of the five wine. 9 points Hedonic scale was used for both quantitative and qualitative evaluation of the products and to find the overall acceptance of wine attributed; Overall acceptability, aroma, color, flavor and taste. The individuals taken were healthy, willing to volunteer and had no history of intolerance to wine.

**Procedure-** All testing was performed one-on-one basis. Judges were brought to sit at the table. All wine samples were coded with numbers and served in a random presentation order (Jackson, 2002). The wines were presented side by side in transparent glasses, and the instruction for the use of the hedonic scale was given. They tested the samples based on the following attributes: color, clarity, smell, taste, body. Responses were given using the 9-point's hedonic scale (Table 8.1). The scale was structured, it had labels but on numbers. Judges tried the first sample and responded by marking on appropriate position on which labels for 9-points hedonic scale were printed. Judges then tasted remaining samples and responded in the same way (Ihekoronye & Ngoddy, 1985). Overall acceptances of the products were judged.

### **3.2.5 Physico-chemical analysis of wine**

#### **1. Determination of alcohol**

In order to perform the physico-chemical analysis, different wines to be analyzed for, were distilled using distillation apparatus. 100ml of wine was transferred in a distillation flask, rinsed with 50ml distilled water, distilled slowly into 100ml volumetric flask. Collected 85-95ml distillate was made up to 100ml with water at 20°C and specific gravity bottle was used to determine the specific gravity. Similarly the distillate of rest of the wine sample was collected and made up to 100ml with water at 20°C. The percentage of alcohol content was estimated by mass using specific gravity table.

#### **2. Determination of volatile acidity**

Volatile acidity as acetic acid was determined using the methods described by Amerine and Ough, 1980. In brief 50ml of wine distillate was titrated against standard 0.05N NaOH using phenolphthalein as an indicator.

Note: 1ml of 0.05 N NaOH is equivalent to 0.003g of acetic acid.

### 3. Determination of esters

Ester as ethylacetate was determined using the methods described by Amerine and Ough, 1980. In brief, 10ml of 0.1N NaOH was added to the neutralized distillate from the volatile acidity determination, and so obtained solution was refluxed on a refluxing apparatus using heating mantle for one hour. Cooled and back titrated the unspent alkali against 0.1 H<sub>2</sub>SO<sub>4</sub>. Taking 50ml of distilled water instead of distilled blank value was determined simultaneously in the same way. The difference in the titer value in ml of standard 0.1N H<sub>2</sub>SO<sub>4</sub> gives the equivalent ester.

Note: 1ml of 0.1N NaOH is equivalent to 0.0088g of ethyl acetate.

### 4. Ripper Titration for Determining Sulfur Dioxide Content in Wine

#### a. Reagent Preparation:

0.01M iodine solution was standardized before use and stored in brown glass in the dark. Solution is stable for 3 months, 1M sodium hydroxide, 25% v/v sulfuric acid, commercially available starch as indicator.

#### Methods

##### To determine free SO<sub>2</sub>:

1. 50.0mL of wine was pipetted into a 250mL conical flask
2. 300mg Vitex (starch) and 5mL of 25% H<sub>2</sub>SO<sub>4</sub> was added to the flask, mixed well.
5. 1g solid sodium bicarbonate added to the flask was followed by titration with 0.01M iodine solution present in the burette.
6. The solution was titrated to a blue color which persists for 30 seconds.
7. The free SO<sub>2</sub> concentration in mg/L was calculated using the following equation.

$$\text{The final equation is: } \text{SO}_2 \text{ (mg/L)} = \frac{(\text{mL iodine}) \times (\text{M iodine}) \times (64) \times (1000)}{\text{mL wine sample}}$$

##### b. To determine total SO<sub>2</sub>:

1. 20.0mL of wine taken into a 250mL conical flask.
2. Then 25mL of 1M sodium hydroxide solution was added.
3. Mixed well and allowed to stand for 10 minutes.
4. 300mg Vitex (starch) and 10mL of 25% H<sub>2</sub>SO<sub>4</sub> was added to the flask. Mixed well.
5. 1g solid sodium bicarbonate added to the flask was followed by titration with 0.01M iodine solution present in the burette.
6. The solution was titrated to a blue color which persists for 30 seconds.

7. The total SO<sub>2</sub> concentration in mg/L was calculated using the equation above.

### **Method for standardizing 0.01M iodine solution against standard sodium thiosulphate solution:**

1. 50mL of 0.01M sodium thiosulphate solution was pipetted into a conical flask.
2. Few mg of Vitex indicator was added.
3. Iodine solution was titrated to a deep blue endpoint which persists for 30 seconds.
4. Reaction was carried out under pH 5; a small addition of acid may be required prior to titration.

Note: Molarity of iodine = 0.25/ (titre in mL)

### **5. Folin-Ciocalteu Micro Method for Total Phenol in Wine**

It is based on the method reported by Slinkard, K.; Singleton, V. L 1997 only the volumes have been reduced.

Gallic Acid Stock Solution: In a 100-mL volumetric flask, 0.500 g of dry gallic acid in 10 mL of ethanol was dissolved and diluted to volume with water. Solution is stable up to two weeks provided kept closed in a refrigerator.

Sodium Carbonate Solution: 200 g of anhydrous sodium carbonate in 800 mL of water was dissolved and stand to a boil. After cooling, few crystals of sodium carbonate were added, and after 24 hour, solution was filtered and made up to 1L.

Calibration curve was prepared by taking, 0, 1, 2, 3, 5, and 10 mL of the above phenol stock solution into 100 mL volumetric flasks, and then diluted to volume with water to obtain phenol concentrations of 0, 50, 100, 150, 250, and 500 mg/L gallic acid. From each calibration solution, sample, and blank, 20 µL pipetted into separate cuvettes, and 1.58 mL water, 100 µL of the Folin-Ciocalteu reagent, was added to each, and then mixed well. Incubated for 30 sec and 8 min at room temperature, and then 300 µL of the sodium carbonate solution was added, and shake to mix. The solution was kept at 20°C for 2 h and absorbance of each solution at 765 nm against the blank (the "0 mL" solution) was determined. Absorbance vs. concentration was plotted. Alternatively, they can be left at 40°C for 30 min before reading the absorbance.

For white wines, 20 µL as for the calibration solutions, but in the case of red wines, diluted the wines by 10 first, then 20 µL was added (or skip the dilution and add 2 µL if you have precise micro pipettors).

Results were reported as total phenols in wines. (Waterhouse, A.L. 1999)

### 3.2.6 Determination of ethanol, methanol, acetaldehyde, higher alcohol, ketones through GC instrument

#### 1. Method for alcohol quantification

##### Column Information

---

Column Name	Rtx-Bac1
Film Thickness	0.32 um
Column length	30.0 m
Inner Diameter	0.32 mm ID
Column Max Temp	240 °C

---

##### Method Description:

Short headspace method for testing.

This is only a sample method for short runs. For analytical work the method parameters must be set according to the specific needs.

Syringe: 2.5ml-HS

01 Headspace01

---

Incubation Temperature (°C)	70
Incubation Time (s)	300
Syringe Temperature (°C)	75
Agitator Speed (rpm)	500
Fill Speed (µl/s)	500
Pull up Delay (ms)	500
Inject to	GC Inj2
Injection Speed (µl/s)	500
Pre Inject Delay (ms)	500
Post Inject Delay (ms)	500
Flush Time (s)	30
GC Runtime (s)	240

---

# CHAPTER FOUR

## RESULTS

### 4.1 Total yeast count

The five *murcha* samples were collected from various parts of the Nepal. The places from where they were collected were given sample code as shown in Table 4.1. The colonies with white, creamy and smooth appearance in the yeast extract peptone dextrose (YEPD) agar medium with 50 ppm chloramphenicol incubated at 28°C for 48 h were counted as the yeast. The number of yeast colony forming units (cfu) per gram of *murcha* samples collected in different places are presented in Table 4.1.

**Table 4.1** Total number of yeasts obtained in YEPD agar plates

Code for Murcha	Yeasts count (cfu/g <i>murcha</i> )
Palpa <i>murcha</i> (PM)	16 X 10 <sup>5</sup>
Lubhu <i>murcha</i> (LM)	11 X 10 <sup>5</sup>
Jorpati <i>murcha</i> (JM)	12 X 10 <sup>5</sup>
Bhojpur <i>murcha</i> (BM)	9 X 10 <sup>5</sup>
Sunsari <i>murcha</i> (SM)	13 X 10 <sup>5</sup>

According to the above Table, the Palpa *murcha* gave the highest yeast count of 16 X 10<sup>5</sup> cfu/g and Bhojpur *murcha* gave the lowest yeasts count of 9 X 10<sup>5</sup> cfu/g.

### 4.2 Morphological characteristics of yeast

**Table 4.2** Study Morphological characteristics of the isolates.

Murcha Code *	Color	Size(dia) (mm)	Shape	Texture	Simple staining	Vegetative reproduction
PM	White creamy	1-3	Circular	Smooth	+ve	Budding
LM	White creamy	1-2	Cir. to oval	Rough	+ve	Filamentous
JM	White creamy	1-2.5	Circular	Smooth	+ve	Budding
BM	White creamy	2 or<	Circular	Smooth	+ve	Budding
SM	White creamy	1.5 or<	Circular	Rough	+ve	filamentous
AP	White creamy	2	Cir. to oval	Smooth	+ve	Budding
RI	White creamy	1-2	Cir. to oval	Smooth	+ve	Budding
GP	White creamy	1-3	Circular	Smooth	+ve	Budding
FW	White creamy	1-2.5	Circular	Smooth	+ve	Budding

Note; Cir, Circular

\* PM, Palpa *murcha*; LM, Lubhu *murcha*; JM, Jorpati *murcha*; BM, Bhojpur *murcha*; SM, Sunsari *murcha*; AP, Apricot; RI, Raisin; GP, Grapes and FW, ghanti phool flower (*Argyrea nervosa*).

The morphological observations of 9 isolates obtained after incubation in YEPD media at 28°C for 48h are shown in Table 4.2. Simple staining followed by microscopic view revealed the above observations. The presented data in the table 4.2 shows that all the colonies are same except two i.e. Lubhu and Sunsari, which shows rough and filamentous reproduction.

### 4.3 Fermentative test of wine yeasts strains

#### 4.3.1 Sugar tolerance test

From the samples collected, 8 yeast strains were isolated from 8 different samples. Isolates that grew on YPD agar plates containing 20% dextrose were selected. The capability of the isolates to grow on medium with high sugar concentrations by tolerating high osmotic pressure was examined further by culturing them in YPD agar plates containing 20%, 25%, and 30% Glucose at 28°C for 24 h and compared the results.

**Table 4.3** Growth at different sugar concentration

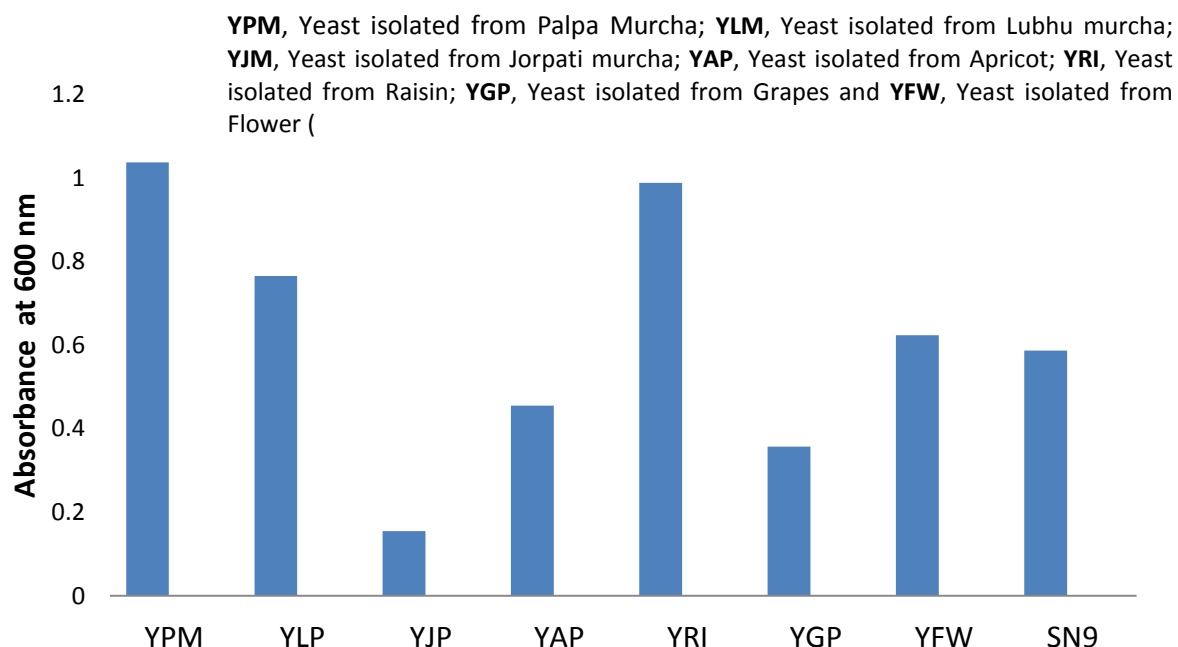
S.N	Sample code*	Glucose concentration(%)		
		20	25	30
1.	YPM	+	+	+
2.	YLM	+	+	+
3.	YJM	+	+	+
4.	YBP	+	+	+
5.	YAP	+	+	+
6.	YRI	+	+	+
7.	YGP	+	+	+
8.	YFW	+	+	+
9.	Com. yeast (SN9)	+	+	+

\* YPM, Yeast isolated from Palpa Murcha; YLM, Yeast isolated from Lubhu Murcha; YJM, Yeast isolated from Jorpati Murcha; YBM, Yeast isolated from Bhojpur; Murcha YAP, Yeast isolated from Apricot; YRI, Yeast isolated from Raisin; YGP, Yeast isolated from Grapes and YFW, Yeast isolated from Flower (*Argyrea nervosa*); Commercial yeast (SN9)

+: positive

### 4.3.2 Sulphur dioxide tolerance test

Tolerance of yeast isolates to sulphur dioxide was examined by culturing the isolates in yeast-peptone-dextrose broth containing 75 ppm SO<sub>2</sub> concentration at 28°C for 24 h, and the growth was compared at wavelength 600 nm. All experiments were conducted in duplicate, and the averages of two readings are presented.

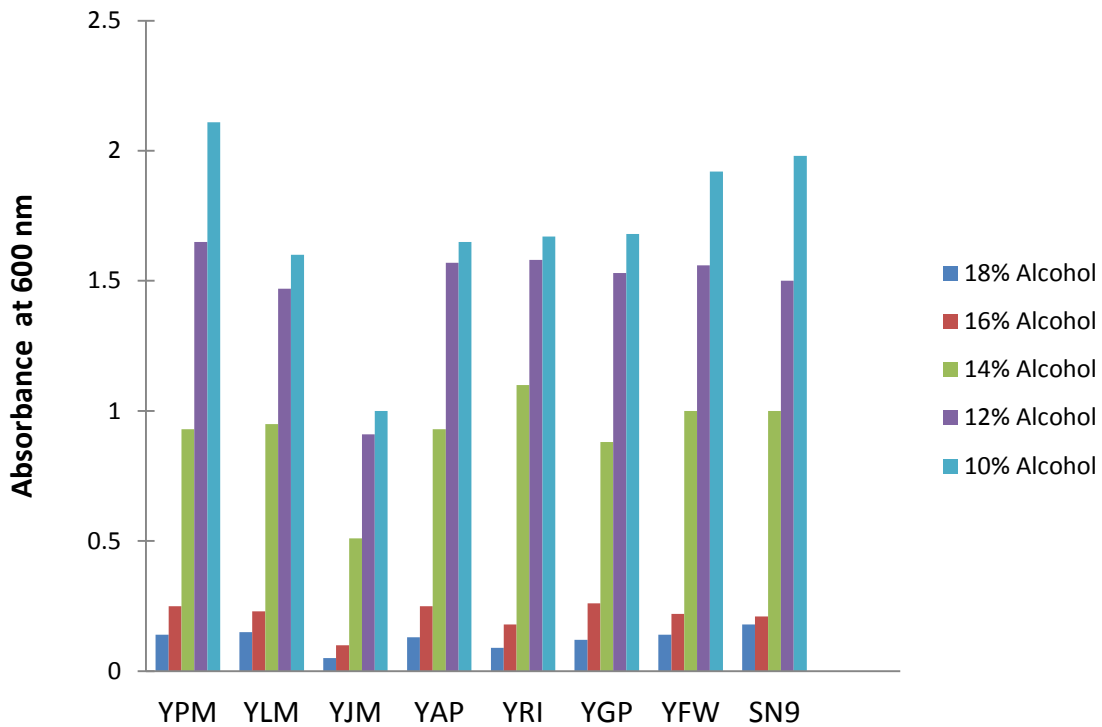


**Fig. 4.1** Growth of yeast on 75ppm Sulphur dioxide containing medium at wavelength of 600nm.

Among all isolate, YPM, YLM and YRI showed better growth in YPD broth containing 75ppm SO<sub>2</sub> than SN9 (commercial yeast). YFW showed similar type of growth with commercial yeast. Yeast isolated from Palpa murcha, Lubhu murcha and Raisin proof that SO<sub>2</sub> tolerance is high as compared with the rest yeast isolates.

### 4.3.3 Alcohol tolerance test

Tolerance of the yeast isolates to alcohol was examined by culturing the isolates in yeast-peptone-dextrose broth (YPD broth) containing 10-18% alcohol at 30°C for 24 h, and the growth was compared at wavelength 600 nm. Eight isolates were selected from different sample based on their ability to form colonies on YPD agar plates containing 12% alcohol and were tested further for their alcohol tolerance in YPD broth with various alcohol percentages 10%, 12%, 14%, 16%, and 18%. All experiments were conducted in duplicate, and the averages of two readings are presented.



**Fig. 4.2** Alcohol tolerance test of yeast isolated from different sources

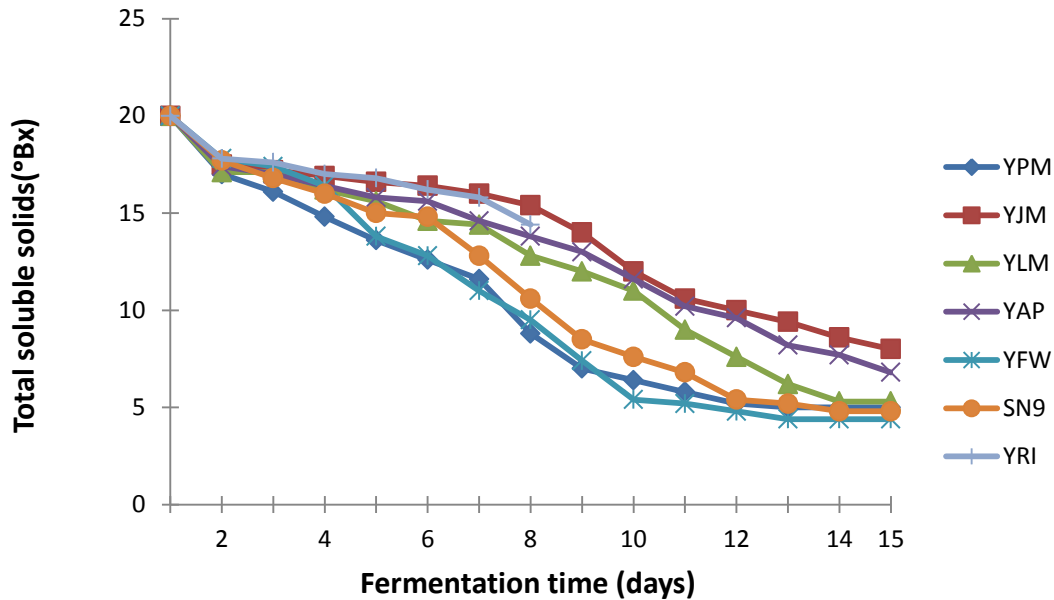
\* YPM, Yeast isolated from Palpa Murcha; YLM, Yeast isolated from Lubhu Murcha; YJM, Yeast isolated from Jorpati Murcha; YAP, Yeast isolated from Apricot; YRI, Yeast isolated from Raisin; YGP, Yeast isolated from Grapes and YFW, Yeast isolated from Flower (*Argyrea nervosa*); SN9, Commercial yeast.

#### 4.4 Test for total soluble solids in YEPD broth

Change in °Brix helps to calculate % of alcohol in fermentation process. In this process °Brix was measured by hand refractometer to calculate the percentage of alcohol produced in fermentation. Approximate amount of alcohol content was calculated by using following formula,

$$\Delta \text{°Brix} \times 0.6 = \% \text{ alcohol content by volume}$$

Total soluble solid changes in 20% glucose containing YEP broth were observed by single loopful inoculation of yeast isolates from different sample. The inoculated culture tube was incubated at 28°C for 15th day and the alcohol content was calculated by measuring change in °Brix at 20°C. All experiments were conducted in duplicate, and the averages of two readings are presented.



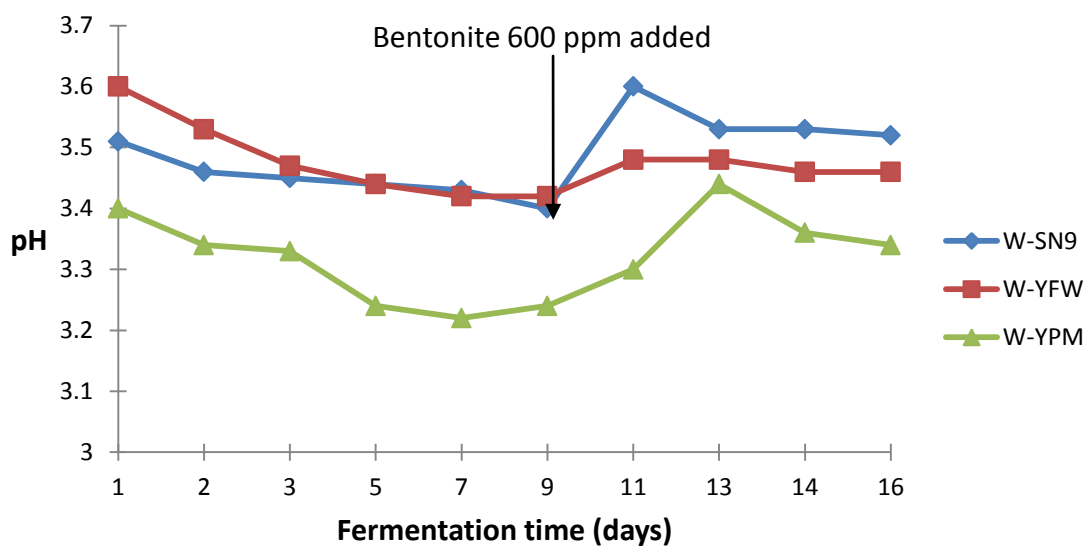
**Fig. 4.3** Total soluble solid changes of YEPD broth by yeast during fermentation

However maximum change in °Brix was observed in YFW from 20 °Brix to 4.4 brix that is alcohol content was approximately around 9.36% alcohol in YEPDA broth. Commercial SN9 yeast, YLM, and YPM showed similar type of change in °Brix.

### 4.5 Chemical analysis of different wines

Three lab prepared wines using two yeast cultures isolated from mucha, flower and one commercial yeast were subjected for pH, acidity and total soluble solids (TSS) test during the fermentation process.

#### 4.5.1 Test for pH

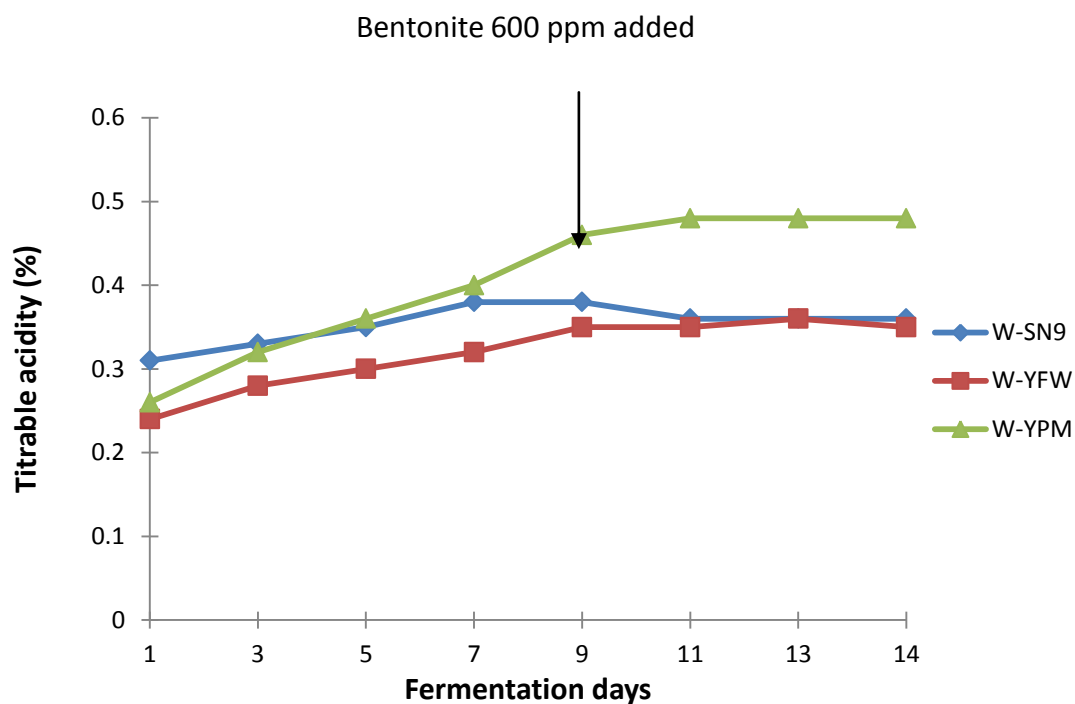


**Fig. 4.4** Changes in pH of fermentable pear juice from yeast from isolated samples and commercial yeast.

# W-SN9, wine made using SN9 yeast; W-YFW wine made using YFW; W-YPM, wine made using YPM.

The pH was measured with a pH meter, an instrument that determines pH quickly and easily. Gradual decrease in pH is observed with increase in fermentation time but when bentonite was added as a clarificant in order to obtain a clear wine. Increase in pH was observed for some time.

#### 4.5.2 Test for acidity

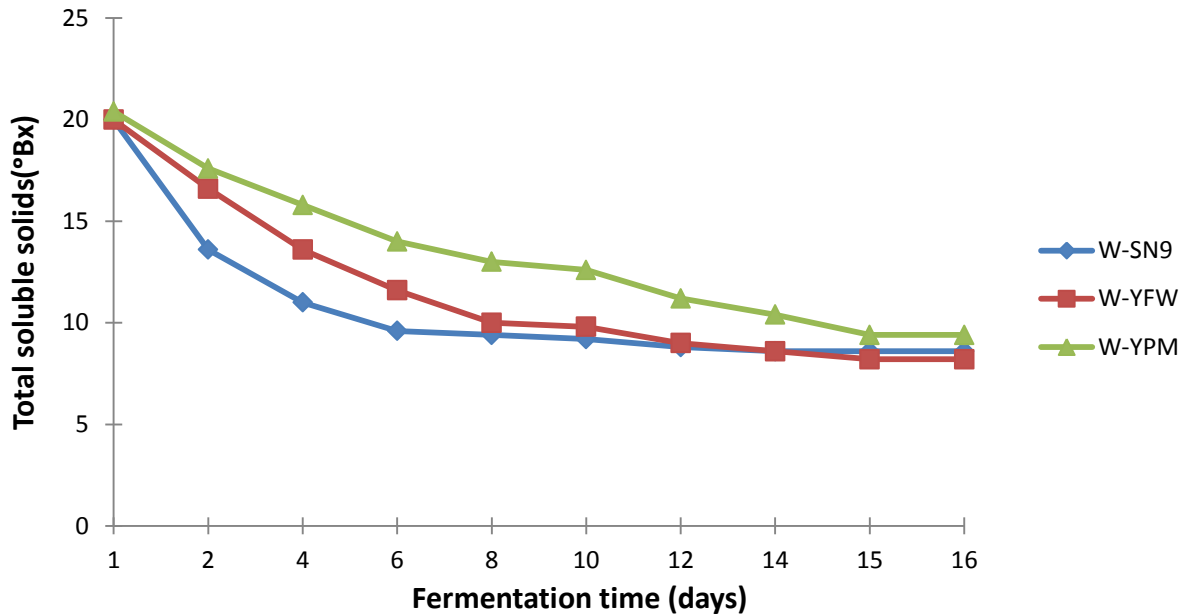


**Fig. 4.5** Titratable acidity of fermentable pear juice from two selected yeast from isolated samples and one commercial yeast.

The titratable acidity of the three wines gradually increased along with the fermentation time. The slight decrease in titratable acidity of fermenting medium was observed with bentonite addition. In the three wines prepared, the titratable acidity of the W-YPM was highest compared to the W-SN9 and W-YFW.

#### 4.5.3 Test for Total soluble solids (TSS) in pear juice

Starter culture added to 5L pear juice containing glass jar was kept to proceed for fermentation. The fermented glass jar was regularly checked for degree Brix by hand-held refractometer. The gradual decrease in degree Brix with fermentation time was observed.



**Fig. 4.6** Change in °Brix of fermentable pear juice from two selected yeast from isolated samples and one commercial yeast.

#### 4.6 Sensory evaluation

The sensory evaluation showed that all wines had an acceptable taste, clarity, sweetness and bitterness but further research is needed to improve the aroma and color of the final product. The results of the sensory evaluation of pear wine and reference wine showed that there were no significant differences in flavor, taste, clarity and overall acceptability between the pear wine and commercial pear wine. However the reference wine received higher sensory rating for color and smell than the pear wine prepared in lab.



**Fig. 4.7** Sensory evaluation of commercial and lab produced wines.

Note: C.W denotes commercial wine, SN9 denotes commercial yeast used to produce wine, W-YFW and W-YPM denotes wines produced from yeast isolated from flower and mucha.

#### 4.7 Physiochemical composition of wine

In the seven wine physiochemical analysis, TSS ranged from 5.8 to 13.8 while TA ranged from 0.29 to 0.48. Compared to the other wines commercial wine 2 possessed the lowest amounts of TSS. However, commercial wine 4 possessed the highest amount of TSS. The wine prepared from the commercial yeast SN9 possessed the lower concentration of total phenol. There was no significant difference in free sulphur dioxide in all wines analysed but significantly high amount of total sulphur dioxide content was perceived in the commercial 3 and 4.

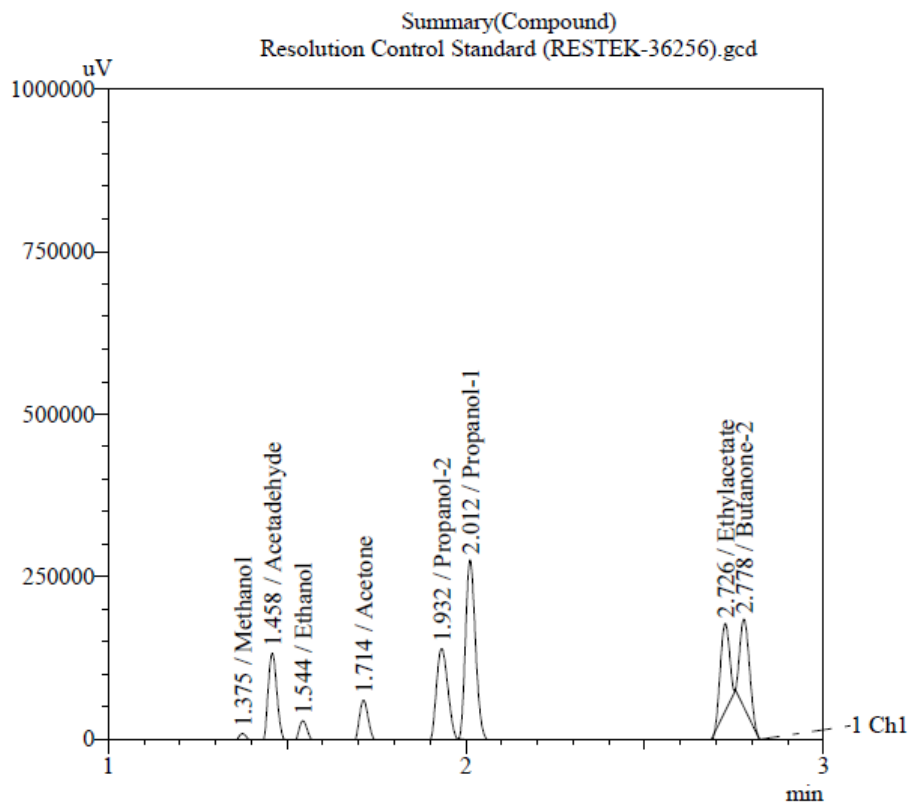
**Table 4.4** Report on different parameters and chemical compounds present in the different variety of wine.

S.N	Sample Parameters	Yeast culture			Commercial wine			
		SN9	YPM	YFW	#1	#2	#3	#4
1.	°BRIX (°Bx)	8.0	9.0	8.2	10.2	5.8	9.2	13.8
2.	Titration acidity (%MA)	0.35	0.48	0.35	0.42	0.32	0.37	0.29
3.	pH	3.55	3.34	3.48	3.43	3.63	3.53	3.72
4.	Alcohol (%abv)	9.17	9.84	10.5	10.5	10.5	10.7	9.3
5.	Volatile acidity(g/L)	0.138	0.132	0.111	0.321	0.444	1.06	0.687
6.	Aldehyde (mg/100mL)	nd	4.62	-	nd	nd	nd	41.27
7.	Total phenol (mg/L)	53	65	75	82	80	98	110
8.	Ester (mg/L)	88	70	113	140	70	281	197
9.	Free SO <sub>2</sub> (mg/L)	2.43	2.54	2.30	nd	nd	5.12	4.5
11.	Total SO <sub>2</sub> (mg/L)	2.43	2.54	2.30	nd	nd	32.64	28
12.	Methanol (mg/100mL)	nd	-	-	nd	nd	nd	13.14
13.	Acetone (mg/100mL)	nd	3.16	-	nd	nd	nd	-

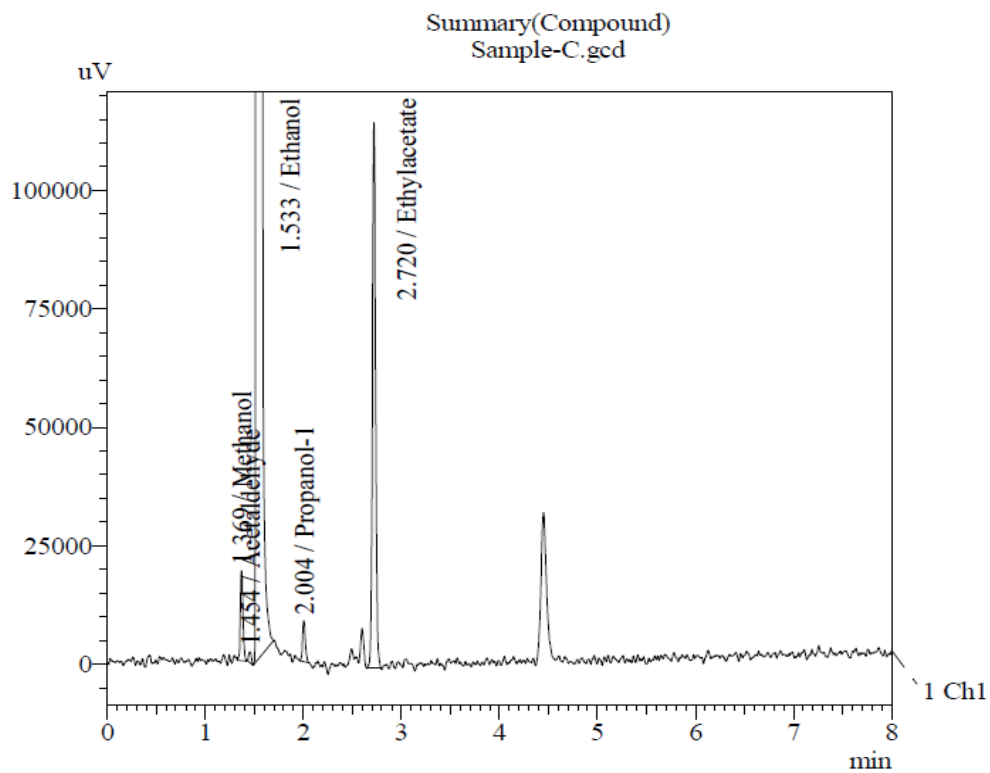
nd, not determined; SN9, commercial yeast used to produce wine; YFW, yeast isolated from ghandi phool flower; YPM, yeast isolated from Palpa murcha

#### 4.8 Analysis of volatiles in different wine

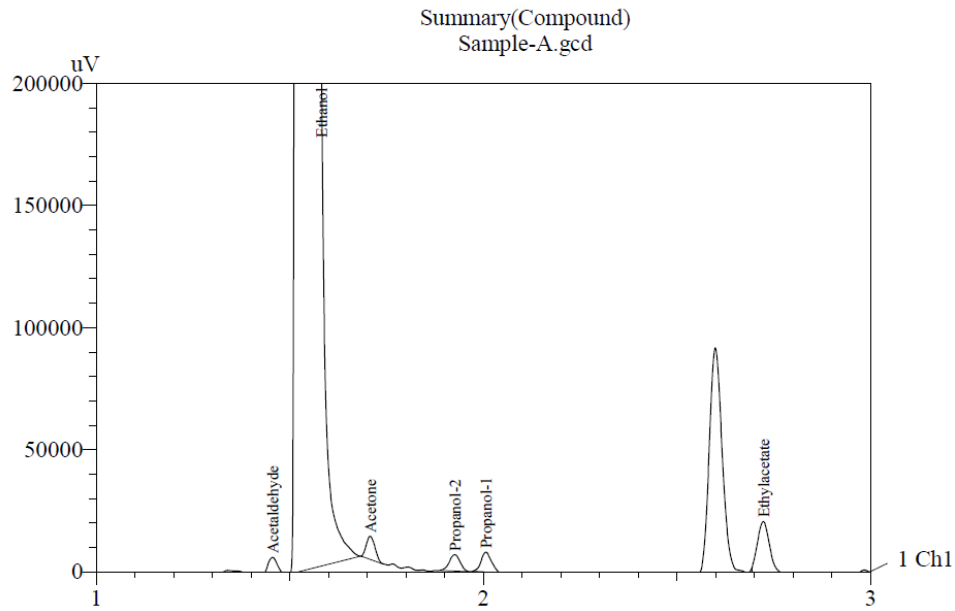
Gas chromatography analysis of the volatiles in three wines CW-4, W-YPM, W-YFW respectively were analyzed to detect the compounds present and to study their significance in wine. Acetaldehydes, propanol-1, propanol-2, ethylacetate, acetone presence were observed in W-YFW and W-YPM. However presence of methanol was detected in C.W-4 along with acetaldehydes, propanol-1, ethylacetate during the qualitative analysis. The analysis was against a standard volatile mix containing eight compounds. The chromatogram of the analyzed samples CW-4, W-YPM, W-YFW against standard volatile mix are as follows;



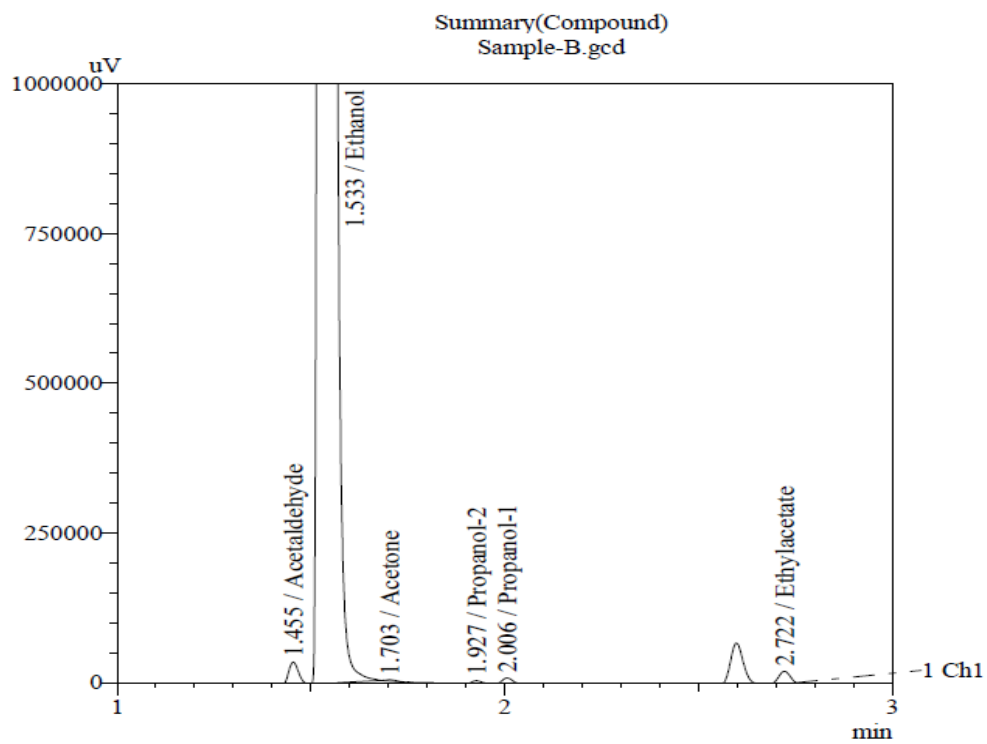
**Fig 4.8** Chromatogram of standard volatile mix.



**Fig. 4.9** Chromatogram of commercial wine



**Fig. 4.10** Chromatogram of wine volatile fermented by the YPM (yeast isolated from Palpa's murcha)



**Fig. 4.11** Chromatogram of wine fermented by YFW (yeast isolated from flower) (*Argyria nervese*).

# CHAPTER FIVE

## DISCUSSION

### 5.1 Total Yeast count

Murcha is a mixed starter culture used in alcohol production and has long tradition in Nepal. The preparation of these starters is mainly confirmed to certain ethnic group of Nepal. As it is prepared without any scientific knowledge, contain mixture of different types of yeast, mold and even some bacteria. . The use of selected pure-culture wine yeasts has improved the quality of wines made, but has reduced the differences between wines. Winemakers are now showing renewed interest in natural fermentations with the objective of producing wines with distinctive flavor and aroma characteristics.

Karki in 1986 isolated *Aspergillus oryzae*, *A.niger*, *Rhizopus sps*, *Mucor*, *Saccharomycopsis fibuligera*, *Saccharomyces cerevisiae* and *Pediococcus* from *murcha*. Since then regular investigations are performed on the traditional alcohol starter *murcha*.

In this research work, the standard plate count was carried out on YEPD agar plates by spread plate method to see the distribution of microorganism per gram of *murcha*. Among five different sample the highest load of yeast was found in *murcha* sample collected from Palpa  $16 \times 10^5$  cfu/gm and lowest in *murcha* sample collected from Bhojpur i.e.  $9 \times 10^5$  cfu/gm. Such variation in total load of microorganism in different *murcha* samples is associated with geographical variation, freshness of the *murcha*, raw material used and the processing technique employed for preparation.

All isolates aseptically cultured on the YEPD agar plates at 28°C 48h were taken and their morphological characteristics were observed. In simple staining, the yeast smear prepared was stained with a single reagent basically stain methylene blue, and observed under microscope for the study of morphological characters. Budding and filamentous were the mostly observed microbes. From the mixed population of organisms only budding yeasts were selected and further identification was carried out on the basis of morphological characteristics and vigorous carbohydrate fermentation. The freshness, raw material used

and the processing technique may be the potential factors for obtaining good fermentive yeast.

## **5.2 Fermentative test of wine yeast strains**

### **5.2.1 Sugar tolerance test**

During wine fermentation process glucose used is released as ethanol and CO<sub>2</sub>. In the fermentation process yeast faces number of stresses such as osmotic pressure causes by high concentration of glucose, ethanol and SO<sub>2</sub> etc., so resultant of such effect is shown by its retarded growth in the fermenting media. Therefore screening of yeast strain, having high osmotic pressure was done. From the murcha and fruit sample collected, 8 yeast strains were isolated and subjected under screening by growing on YPD agar plates containing 20% dextrose. Yeast that grew on 20% dextrose containing media was selected. The capability of the isolates to grow on medium with high sugar concentrations by tolerating high osmotic pressure was examined further by culturing them in YPD agar plates containing 20%, 25%, and 30% at 28°C for 24 h. Among all isolates, yeast isolated from Palpa murcha (YPM) and Raisin (YRI) showed better growth in YPD agar plates containing 20%, 25% and 30% dextrose, than the commercial strain (*Saccharomyces Bayanus* SN9), which is widely used for wine fermentation. Likewise, YLM showed similar type of growth at 30% dextrose with the commercial strain (*Saccharomyces Bayanus* SN9).

Growth inhibitors such as organic acids, aldehydes, higher alcohols, and fatty acids produced by yeasts may have accumulated during fermentation. Recently, it has been shown that octanoic and decanoic acids, byproducts of alcoholic fermentation, act in synergy with ethanol. Their presence, even at low concentrations occurring in beer and wine, could explain the higher toxicity of ethanol produced during fermentation. (Viegas et al 1986). The fermentation of sugars in the brewing industry is generally carried out by employing yeast producing 4 to 9% (w/v) ethanol. Any increase in ethanol concentration during fermentation, up to certain limits, would be desirable both from quality and economic considerations. Furthermore, increased ethanol yields are important in industrial ethanol production. However, high substrate concentrations have been shown to inhibit yeast growth and fermentation as a result of high osmotic pressure and low water activity. (Jones et al 1981).

From this data we can conclude that all the yeast isolates have the ability to grow and carry out fermentation in the concentration of below and at 30% dextrose concentration, only the rate of efficiency of fermentation differs. YPM (Yeast isolated from Palpa murcha) and YRI (Yeast isolated from Raisin) has better sugar tolerance than the rest of the isolated yeast.

Thus, we can conclude that high substrate concentrations have been shown to inhibit yeast growth and fermentation as a result of high osmotic pressure and low water activity with minimum effect on YPM (yeast for palpa murcha) and YRI (yeast for raisin).

### **5.2.2 Sulphur dioxide tolerance test**

The microflora of fermentation can be controlled using sulphur dioxide, which is highly toxic to most non-Saccharomyces yeasts, while ethanol production strains of Saccharomyces in general are quite resistant to SO<sub>2</sub> and susceptibility to sulphite varies widely. Thus, the effects of sulphite can be selective by reducing the diversity of yeast strains. During wine preparation there are several non-saccharomyces yeasts on the surface of substrate. Inoculation with a commercial starter culture avoids risk of spoilage, and provides a shorter fermentation time and better, more predictable wine flavour. Generally, 75ppm SO<sub>2</sub> is used to prevent the growth of non- saccharomyces yeast, and gradually support the starter culture growth.

SO<sub>2</sub> addition in the wine fermentation is a common practice because of role its significant relation with aldehydes. Aldehydes are byproducts of fermentations that contribute to a wine's flavor. Besides their role in flavor, aldehydes also play a role in color, by reacting with sulfites and preventing bleaching, and more importantly, by participating in the binding of anthocyanins to tannins and stabilizing color. Finally, aldehydes also play a role in texture, due to the above participation in tannin polymerization reactions. SO<sub>2</sub> affects the enzyme that converts acetaldehyde into ethanol (aldehyde dehydrogenase). SO<sub>2</sub> also binds directly with acetaldehyde, preventing its transformation into alcohol. Therefore SO<sub>2</sub> presence in the wine prevents aldehydes transformation and in return aldehyde preserves the wine flavor, color, clarity, texture etc. (Frivik and Ebeler, 2003). SO<sub>2</sub> plays a significant role in wine preparation; hence its presence is important to assure good wine quality. The reason for not adding SO<sub>2</sub> in the wine prepared is to make wine chemical free and available in its pure form as organic wine.

### **5.2.3 Alcohol tolerance test**

Higher ethanol concentration has three major effects on yeast cells. It inhibits cell growth, cell viability, and fermentation, although to different extents. One of the major target sites of ethanol is the plasma membrane of yeast and other microorganisms, as well as the membrane of the various cellular organelles. The damage caused by ethanol to the cell membrane results in altered membrane organization and permeability (Thomas and Rase, 1979).

However for example, in a study with a sake yeast strain, growth was completely suppressed by 12% (w/v) ethanol, but at the same concentration, the fermentation rate was still 25% of the control rate, with fermentative capacity still detected up to 30% (w/v) ethanol. Thus, the fermentation rate is the most ethanol tolerant of the three parameters (Brown et al., 1981).

Generally, light wines, such as red, white, and sparkling wines, contain less alcohol (10-14%). None of the isolates or the commercial strain grew on YPD agar plates containing 20% alcohol. Eight isolates were selected from different sample based on their ability to form colonies on YPD agar plates containing 12% alcohol and were tested further for their alcohol tolerance in YPD broth with various alcohol percentages 10%, 12%, 14%, 16%, and 18%. Generally, the isolates did not grow well in YPD broth containing above 14% alcohol. Isolates with higher alcohol tolerance were YLM, YFW, YGP, and YPM, and showed better growth than SN9 (commercial yeast) in 10% alcohol. YPM and YLM showed better growth than SN9 (commercial yeast) in 12% alcohol. In 14% alcohol isolates showed similar type of growth with SN9 (commercial yeast). At 14% alcohol concentrations, ethanol must have toxic effect to the yeast cell that resulted in the retarded growth of all.

#### **5.2.4 Test for total soluble solids in YEPD broth**

Change in °Brix helps to calculate % of alcohol is one of the important elements during fermentation process. Fermentation of YEPDA broth by strain of yeast at 28°C containing initially 20°Brix solution up to 14th day and the alcohol content was compared by measuring change in °Brix at 20°C. In figure 4.3 continuous decrease in °Brix is observed up to 12th day of fermentation and little or insignificant change is noticed afterwards in °Brix were similar that means further incubation does not increase the content of alcohol or pushes fermentation ahead. The reason may be due to inhibition of cell growth and viability with increasing ethanol concentrations, resulting in inhibition of fermentative capacity, or nutrient deficient in the fermentative medium.

#### **5.5.1 Test for total soluble solids in pear juice**

A gradual decreasing trend of residual degree Brix was recorded at specific interval of 48 hour throughout the fermentation process up to 12 irrespective of treatment indicating the use of fermentable sugar by all the three yeasts. The rate of fermentation was quite higher during first 4 days then gradually it declined till the end in W-SN9, W-YPM and W-YFW as shown in fig.4.6. However maximum decline in TSS was observed in case of W-SN9 followed by W-YFW and W-YPM. A gradual decrease in TSS is a typical nature of fermentation where due to low alcohol production initially, the rate of fermentation was higher and further absence of decrease in the degree Brix may be due to inhibition of cell growth and viability

with increasing ethanol concentrations, according to Henschke (1997) vitamin deficiencies occurring due to juices treated with high concentrations of SO<sub>2</sub>, pasteurization or ion exchange must have resulted in sluggish and stuck fermentations. However, Blateyron & Sablayrolles (2001) used one hundred and seventy eight difficult-to-ferment musts, selected by enologists from different regions in France, to perform fermentation trials. They effectively fermented all sluggish and stuck fermentations by adding 7 mg/L oxygen and 300 mg/L DAP at the halfway point of the fermentation process.

## **5.6 Sulphur dioxide analysis**

Only White wines were subjected to the analysis for the SO<sub>2</sub> presence and red wines were not analyzed due to the procedure followed for the red wine was different. SO<sub>2</sub> found in the lab prepared wine were significantly low as compared to the wine available in the market. This is because addition of SO<sub>2</sub> in the commercially available market was more and they might have used in order to control the unwanted microbial load in the wine. Free SO<sub>2</sub> content in all the wine were between 2 to 5mg/l were as the total SO<sub>2</sub> content were very high in the commercially available wine. In lab prepared wine SO<sub>2</sub> found was produced by yeast due to interaction with the must whereas the SO<sub>2</sub> found in the commercial wine may have been externally added as a preservative.

Nevertheless, high levels of SO<sub>2</sub> in must could play a part in stuck/sluggish fermentations as *S. cerevisiae* accumulates SO<sub>2</sub> very rapidly due to the higher pH inside the cell than in the suspension. Inside the cell SO<sub>2</sub> causes a rapid decrease of intracellular ATP levels which results in cell death. It is also known that SO<sub>2</sub> reacts with NAD<sup>+</sup>/NADP, affect enzyme systems as well as cleave thiamine and disulphide bridges of proteins (Alexandre & Charpentier, 1998).

## **5.7 Physicochemical characteristics of wine**

The physicochemical characteristics of the wines prepared with different yeast strains clearly show that TSS of all the wines ranged from 8.0 to 9.4°B and all the wines were fermented to dryness as the residual concentrations of total sugars of all the wines was less than 0.5%. Although the TSS of the wines was quite high, the residual sugar content was very low. It is because of the fact that TSS includes all soluble components including sugar. The rate of fermentation showed that the SN9 and YFW strains gave the highest reduction in TSS and YPM gave the lowest. The titratable acidity of the wines prepared with different yeasts ranged between 0.35 to 0.48 which was virtually comparable in all the wines. Thus, yeasts did not influence the acid production in the wines and is desirable. The yeast strain suitable for wine production should have high fermentability, tolerance to ethanol, sedimentation property and no effect on titratable acidity.

Further, the pH of wines was according to their respective acidities. The above table reveals that the W-YFW and W-SN9 produced higher ethanol (10.5% and 9.84% respectively) than other strains, which is of great interest to produce high quality wine. The amount of ethanol produced by the yeast is desirable character and YFW strain proved to be the best. Further, all the three strains (YFW, SN9 and YPM) consumed practically all the initial sugar, one of the most important criteria for selecting yeast for wine making. The results shown also reveal that YFW strain had the highest fermentation efficiency followed by SN9 strain. However, YPM strain gave minimum fermentation efficiency. Although, the yeast fermented wines to almost dryness their rate of fermentation were different. This is due to the fact that different yeast strains have different fermentation efficiency to produce varying level of ethanol and other by- products. It is clearly evident that YFW strain produced the maximum aldehyde content; strain YPM produced the minimum aldehyde content in the pear wines and finally no aldehyde content was found in the commercial pear wine. Production of low level aldehyde is a desirable characteristic of wine yeast. Generally, production of aldehyde indicates incomplete alcoholic fermentation or oxidation of alcohol. The content of methanol in different wines was comparable. The highest amount of methanol was produced by commercial pear wine while the nil was recorded in wines fermented with YFW and YPM strain. Production of methanol is an undesirable characteristic of wine yeast; the strains of *S. cerevisiae* generally do not produce. Study further revealed that out of various parameters studied titratable acidity, pH, total sugars, ethanol and colour units did not contribute much to the separation of yeast. Thus, based on physicochemical analysis, hydrolysis of pectin present in fruits might have contributed to production of methanol or Dimethyldicarbonate (DMDC) an effective pre- bottling sterilant, use of DMDC is important in stabilizing lower alcohol products from additional fermentation in the bottle, and also allows a reduction in the quantity of sulphur dioxide. DMDC breaks down rapidly in wine, producing carbon dioxide and leaving methanol at very low levels not harmful to health. Nevertheless, the amount of methanol in the commercial pear wines was in low amount to be significant. The physicochemical analysis of the data clearly states that YFW, YPM and SN9 strains have many similar characteristics.

### **5.7.1 Acidity in wine**

The amount of volatile acid as acetic acid was in the range between 0.11 to 0.13 g/l obtained from the wine prepared in the biotechnology lab. However the commercial wine test showed a different result of 0.33 to 1.06 g/l of acetic acid, a significantly high amount was observed may be due to the high stress response to the yeast during fermentation because of high sugar presence. However none of the wine exceeded the upper limit of acetic acid prescribed by international wine consultants as below. European Union upper

limit of acetic acid is 1.08g/l for white wines and Australian limits no more than 1.5g/l of volatile acidity expressed as acetic acid (NWWWS, 2009).

### **5.7.2 Aroma compounds in wine**

Aromatic compound ester as ethyl acetate and phenols tested in the wine were between 88-281 mg/L and 53-110 mg/L respectively obtained through titration methods. Ethyl acetate being an only compound tested in the wine may not however give the overall value of aromatic compounds present in the wine. Thus the obtained values are compared with the commercial wine and noticed the difference in the value. The difference may be the result of the combined use of fruits the other additive in the commercial wine that have resulted in the produce of high esters and phenol.

Chen (2016) found 3-methyl-1-butanol (7.18 %), ethyl acetate (4.5 %), 1-nonanol (4.16 %), 1-hexanol (3.98 %), 2-methyl-1-propanol (3.88 %), formic acid (2.57 %), 1-octanol (2.57 %), phenyl ethyl alcohol (2.29 %), 2-hydroxy-ethyl propanate (2.2 %), 2-methoxy-4-methyl-phenol (1.74 %) etc. as dominant components in Xinjiang Kuerle Fragrant Pear Wine when analyzed by combined gas chromatography/mass spectrometry with PA and PDMS fibers. (Chen, 2016). 23 volatile compounds has been isolated and identified successfully in sand pear juice as Shisho and Atago by capillary gas chromatography-mass spectrometry (GC-MS). It's been found out that the main components in Shisho juice are 2-Nonanone (23.08%), Propanoic acid ethyl ester(19.51%), Butanoic acid butyl ester(10.32%), ethanol (9.82%), Ethyl acetate (8.53%), Anthraoene (4.68%), Acetic acid (4.41%), Cyclohexene (4.29%) etc., and that the main components in Atago juice are Propanoic acid, 2-methyl-ethyl ester(32.53%), Ethyl acetate(24.56%), Isopropyl alcohol(17.81%), 1-Undecanol (16.02%) and Butanoic acid butyl ester(4.59%) (Xie, 2014).

### **5.8 Sensory analysis of wine**

Two reference and laboratory prepared three wine were evaluated under the panel of six judges. Color, clarity of the reference wine were rose and cloudy whereas the prepared lab wine had faint rose color and have clear clarity. Reference wine gave strong fruity and spicy aroma than the prepared lab wine. Reference wine was sweeter compared to the lab wine which is comparably less sweet. Similarly acidity, dry sensation or bitter taste and density of all the reference and lab wine were almost same i.e., low acidity, low dry sensation and provided thin density. The sensory analysis data shows that appearance, colour, volatile acidity, overall impression, total acidity have contributed towards separation of yeasts into two groups, group I comprising of YFW and SN9 and group II comprising of YPM. Color, body, sweetness and volatile acidity apparently have not been affected by the type of yeast. Out of various characteristics; astringency, bitterness, titratable acidity are related with

group I positively while, astringency, bitterness, titratable acidity are closely associated with group II.

Finally the overall impression of the two reference wine and three laboratories made wine were almost acceptable with similar characteristics. Therefore from the above data we came to conclude that the wine prepared in the laboratory was almost similar or acceptable with the reference wine.

## CHAPTER SIX

### CONCLUSION AND RECOMMENDATION

#### 6.1 Conclusion

Five types of *Murcha* collected from different altitudes and regions of the country, flower (*Argyrea nervosa*) from Kritipur and fruits from local markets were sampled. Screening and isolation of brewing yeasts, fermentation of fresh pear juice by the isolated brewing yeasts, and lastly study of different parameters of the produced wine were carried out. Thus, the total work was divided into three phases.

1. In the first phase, isolation was done on fresh YEPD agar petri plates by pour plate and direct method from the *murcha* and fruits collected from various parts of Nepal.
2. In the second phase, these isolates were subjected to total yeast count, identification based on standard microbiological procedure, ability of different yeast strains to produce ethanol and their tolerance to sugar, SO<sub>2</sub>, ethanol was successfully investigated for the selection of brewing yeasts.
3. The selected brewing yeasts were used to develop wine in the lab and comparative study was carried out with wine prepared from commercial *Saccharomyces bayanus* (SN9). Sensory attributes of wine of wine prepared from yeast isolates were comparable to wine prepared from commercial strains. Therefore these strains may have industrial significance and represents an interesting candidate for use as wine yeast. Further, comparative analysis between lab prepared wine and commercial wine resulted in the significant similarity in body and taste however color, aroma and sweetness differ significantly.
4. It may be concluded that 14 day fermentation of pear juice carried out by yeast isolated produced the quality wine. The best biochemically active strain YFW was found to produce high alcohol wine from pear fruits compared to YPM and SN9. In the optimization parameters for the alcohol fermentation using pear, the concentration of alcohol 10.35% (v/v) achieved was the highest. The pear wine was light pink after clarified and with a pleasant fruit aromas. This may provide some technical reference for the industrial production of the pear wine.
5. Fermentative yeast isolated from *murcha*, different fruit and flower were found to be applicable to ethanol production. These isolates could contribute for the improvement of the wine quality and also could be used to create an identity for the wine produced in Nepal. It can be concluded that Nepali mountain pear grown in Pharping of Kirtipur are suitable for winemaking.

6. The physicochemical analysis of the lab prepared wines had comparatively lower amount of volatile acidity, total phenol, ester and total SO<sub>2</sub> than the commercial Nepalese wine. The presence of these compounds in high concentration may have added additional aroma, taste and color to the commercial wines. The presence of methanol in one of the commercial wine found might confer moderate toxicity to the consumers.
7. Series of studies conducted focused on the yeast responsible for winemaking. Although the microbiological and biochemical evaluation of the yeast strain in these studies permitted the scientific selection of strains with good winemaking properties. Furthermore research is needed to improve the standard and methodology in the preparation procedure of the pear wine to improve its standard.

## 6.2 Recommendations

After completing this research work following suggestions is forwarded to people who might be further interested in working on pear wine from yeast isolates.

1. Study on *murcha* and different samples such as flower, fruits with a high possibility of having a presence of wine yeast should be carried out in order to discover noble yeast.
2. Beverages products are of great value to the country economy. Isolation of more potent wine yeast microorganisms from indigenous fermenting starter culture such as *murcha* as well from substrate such as fruits, flower and their effective use to add a value to the product through fermentation should be done to contribute for peoples benefit.
3. The yeast strains isolated from *murcha*, and flower should be identified at species level and their novelty if established could replace the commercial strains imported from abroad. It necessitates the need of molecular identification of yeast isolates based on 16srRNA sequencing, since morphological characteristic and identical biochemical properties of yeast isolates doesn't allow the accurate confirmation.
4. Moreover there is necessity of identifying the physicochemical present in the wine more accurately. Further research work has to be made to enhance and incorporate the quantity of the compounds through improvement in the processing technology to impart good flavor, aroma, color and taste to the wine.
5. Tonnes of apples, pears and other fruits are being wasted every year; fermentation technology or some alternative method must be applied to save the loss.
6. Nepal is rich in medicinal herbs, these herbs if applied in juice fermentation as oak chips will bring drastic change in the dimension of wine.
7. A laboratory dedicated to the fermentation technology must be established with a view to solve the economic problem and to increase the number of skilled manpower in the field of fermentation technology.

## REFERENCES

- Abe K, Saito T, Terai O, Sato Y & Kotobuki K, (2008). Genotypic difference for the susceptibility of Japanese, Chinese and European pears to *Venturia nashicola*, the cause of scab on Asian pears. *Plant Breeding*; **127**:407–412.
- Adams M R, (1990). Tropical aspects of fermented foods. *Trends Food Sci. Tech.*, vol.1:141-144.
- Akubor P I, Obio S O, Nwodomere K A & Obiomah E E, (2012). Production and Quality Evaluation of Banana Wine. *Plant Foods for Hum. Nutr.*, **58**: 1–6.
- Álamo M, Nevares D, Gallego I, Martin C & Merino S, (2008). Aging markers from bottled red wine aged with chips, staves and barrels. *Analytica Chimica Acta*, **621**:86-99.
- Alexandre H & Charpentier C, (1998). Biochemical aspects of stuck and sluggish fermentation in grape must. *J. Indu. Microbiol. Biotechnol.*, **20**:20-27.
- Ali Azam S, (2008). For Practical Action in food processing and nutrition with small-scale processors in developing countries. Website: <http://www.practicalaction.org/>
- Amerine M A & Ough C S, (1980). Methods for analysis of musts and wines. New York Wiley-Interscience.
- Arfaioli P & Bosetto M, (1993). Time changes of free organic acid content in seven Italian pear (*Pyrus communis*) varieties with different ripening times. *Agriculture Med*, **123**:224-230.
- Arribas M V & Polo M C, (2005). Wine making biochemistry and microbiology: *Curr. Knowled. Future Trends. Food Sci. Nutr.*, **45**:265-86.
- Bailey L H, Bailey E Z & the staff of the Liberty Hyde Bailey Hortorium (1976). *Hortus third: A concise dictionary of plants cultivated in the United States and Canada*. New York: Macmillan.
- Bajaj B K & Bajpai P, (2011). Selection and characteristics of yeast for desirable fermentation characteristics, **vol.2**:107-109.
- Balasubramaniam V M, Farkas D & Turek E J, (2008). Preserving food through high pressure processing, *Food Tech.*, **11**:33-38.

Barrio-Galán R D, Pérez-Magariño S & Ortega-Heras M, (2011). Techniques for improving or replacing aging on lees of oak aged red wines: the effects on polysaccharides and the phenolic composition. *Food Chem.*, **127**:528-540.

Baslingappa S S, Thakor N J & Divate A D, (2014). Department of Agricultural Process Engineering, College of Agricultural Engineering and Technology, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli-415712, Dist Ratnagiri (MS), India.

Baumann A R, Martin S E & Feng H, (2005). Power ultrasound treatment of listeria monocytogenes in apple cider, *J. Food Proto.*, **68(11)**:2333-2340.

Bautista-Ortín A, Lencina A, Cano-López M, Pardo-Mínguez F, López-Roca J. & Gómez-Plaza E. (2008). The use of oak chips during the ageing of a red wine in stainless steel tanks or used barrels: effect of the contact time and size of the oak chips on aroma compounds. *Aus. J. Grape and Wine Res.*, **14**:63-70.

Berg H W & Akiyoshi M, (1956). Some factors involved in browning of white wines. *Ame. J. Enol. Vitic.*, **7**:1-7.

Berry C J, (2000). *First Steps in Wine Making*. Published by G. W. Kent, Inc. 3667 Morgan Road, Ann Arbor MI 48108, 235.

Beveridge T & Rao M A, (1997): Juice extraction from apples and other fruits and vegetables, *Crit. Rev. Food Sci. Nutr.*, **37(5)**: 449-469

Bintsis T, Litopoulou-Tzanetaki E & Robinson R K, (2000). Existing and potential applications of ultraviolet light in the food industry- a critical review, *J. Sci. Food Agri.*, **80**:637-645.

Bisson L F & Butzke C E, (2000). Diagnosis and rectification of stuck and sluggish fermentations. *Ame. J. Enol. Vitic.*, **51**:168-177.

Blake A, Kotseridis Y, Brindle I D, Inglis D & Pickering G J. Effect of light and temperature on 3-alkyl-2-methoxypyrazine concentration and other impact odourants of Riesling and Cabernet Franc wine during bottle ageing. *Food Chem.*, **119**:935-944.

Blateyron L & Sablayrolles J M, (2001). Stuck and slow fermentations in enology: Statistical study of causes and effectiveness of combined additions of oxygen and diammonium phosphate. *J. Biosci. and Bioeng.*, **91**:184-189.

Board R G, (1983). *A Modern Introduction to Food Microbiology*. Oxford, UK: Blackwell Scientific Publications.

Boulton R B, Singleton V L, Bisson L F & Kunkee R E, (1995). Principles and Practices of Winemaking. Chapman & Hall, New York.

Braddock R J, (1999). Single-strength orange juice and concentrates, in R. J. Braddock (Ed.), Handbook of citrus by-products and processing technology, Wiley, New York, 53-83.

Brown S W, Oliver S G, Harrison D E F, & Righelato R C, (1981). Ethanol inhibition of yeast growth and fermentation: differences in the magnitude and complexity of the effect, Euro. J. App. Microbiol. Biotechnol., **19**: 151.

Bruce W Z, Kenneth C F, Barry H G, Fred S N (1995). Fining and Fining Agents. Wine Analysis and Production, 242-247.

Cassano A, Drioli E, Galaverna G, Marchelli R, Di Silvestro G & Cagnasso P, (2003). Clarification and concentration of citrus and carrot juice by integrated membrane process, *J. Food Engin.*, **57**: 153-163.

Castriota-Scanderbeg A, Hagberg G E, Cerasa A, Committeri G, Galati G, Patria F, Pitzalis S, Caltagirone C & Frackowiak R, (2005). The appreciation of wine by sommeliers: a functional magnetic resonance study of sensory integration, **25**:570–578

Chassagne D, Guilloux-Benatier M, Alexandre H & Voilley A, (2005). Sorption of wine volatile phenols by yeast lees. Food Chem., **91**:39-44.

Chen J L, Wang Z F, Wu J H, Wang Q & Hu X S. 2007 Chemical compositional characterization of eight pear cultivars grown in China. Food Chem., **104**:268–275.

CHEN Ji-luan, Ji-hong W U, FENG Zuo-shan, Yong-kun M A & Xiao-song H U, (2016). Analysis of Volatile Components in Xinjiang Kuerle Fragrant Pear Wine.

Cho J Y, Kim C M, Lee H J, Lee S H, Cho J A, Kim W S, Park K H & Moon J H. 2013 Caffeoyl triterpenes from pear (*Pyrus pyrifolia* Nakai) fruit peels and their antioxidative activities against oxidation of rat blood plasma. *J. Agri. Food Chem.*, **61**:4563–4569.

Colonna A, Adams D O, & Noble A C, (2004). Comparison of procedures for reducing astringency carry-over effects in evaluation of red wines. *Aus. J. Grape and Wine Res.*, **10**: 26–31.

D'Amico D J, Silk T M, Wu J & Guo M, (2006). Inactivation of microorganisms in milk and apple cider treated with ultrasound. *J. Food Proto.*, **69(3)**: 556-563.

Devkota L N, (1999). Deciduous fruit production in Nepal. <http://www.fao.org/docrep/004/ab985e/ab985e09.htm>.

Dickinson J R & Schweizer M, (2013) editors. The Metabolism and Molecular Physiology of *Saccharomyces cerevisiae*. Philadelphia, PA: Taylor & Francis, **59**:1-5.

Dirar H, (1992). Sudan's Fermented Food Heritage in Applications of Biotechnology to Traditional Fermented Foods. Washington (DC): *National Academies Press*.

Dittmer P R & Desmond J, (2005). *Principles of Food, Beverage, and Labor Cost Controls*. Hoboken, NJ: John Wiley and Sons, **3**:75-86.

Duarte W F, Dias D R, Oliveira M J, Teixeira J A, Silva J D & Schwan R F (2014). Characterization of different fruit wines made from Cocoa, Cupuassu, Gabiroba, Jaboticaba and Umbu. *Food Sci. Technol.*, **30**:1-9.

Dunn B, Levine RP & Sherlock G. 2015 Microarray karyotyping of commercial wine yeast strains reveals shared, as well as unique, genomic signatures. *BMC Genomics*, **6**: 53.

Eustis R H, (2010). System for non-deleterious accelerated aging of wine or spirits. 11/856,893. USA Patent Application.

Ferreira D, Guyot S, Marnet N, Delgadillo I, Renard C M & Coimbra M A (2002). Composition of phenolic compounds in a Portuguese pear (*Pyrus communis* L. var. S. Bartolomeu) and changes after sun-drying. *J. Agric. Food Chem.*, **50**: 4537–4544.

Fleet GH & Heard G, (1993). Yeasts-growth during fermentation. *Wine Microbiol. Biotechnol.*, (Fleet GH, ed).

Frivik S & Ebeler S, (2003). "Influence of sulfur dioxide on the formation of aldehydes in white wine". *Am. J. Enol. Viti.*, **54(1)**:31-38.

Fundira M, Blom M, Pretorius I S & Van Rensburg P, (2012). Selection of yeast starter culture strains for the production of marula fruit wines and distillates. *J. Agri. Food Chem.*, **50(15)**:35-42.

Galvis-Sánchez A C , Gil-Izquierdo A & Gil M I, (2003). Comparative study of six pear cultivars in terms of their phenolic and vitamin C contents and antioxidant capacity. *J. sci. food Agri. and Food Chem.*, **83**:995–1003.

Garde-Cerdán T, Marsellés-Fontanet A R, Arias-Gil M, Ancín-Azpilicueta C & Martín-Belloso O, (2008). Effect of storage conditions on the volatile composition of wines obtained from

must be stabilized by PEF during ageing without SO<sub>2</sub>. *Innov. Food Sci. and Emerging Technol.*, **9**: 469-476.

Godoy A, Herrera T & Ulloa M, *Más allá del pulque y el tepache: Las bebidas alcohólicas no destiladas indígenas de México*. Mexico: UNAM, Instituto de Investigaciones Antropológicas, 2003. nson, H. *vintage: The Story of Wine*. Simon & Schuster. pp. 11–6. ISBN 0-671-79182-6.

Gómez-Plaza E & Cano-López M, (2010). A review on micro-oxygenation of red wines: claims, benefits and the underlying chemistry. *Food Chem.*, **125**:1131-1140.

Gotame T P, Paudyal K P, & Khatiwada P P, (2014). Status of Fruit and Nut Genetic Resources in Nepal, Indigenous and Exotic Varietal Inventory. Nepal Agricultural Research Council, National Agriculture Research Institute, Horticulture Research Division, Khumaltar, Lalitpur, Nepal.

Gould G W, (2001). Symposium on 'Nutritional effects of new processing technologies' – New processing technologies: an overview, *Proceed. Nutr. Society* **60**: 463-474.

Gresele P, Cerletti C, Guglielmini G, Pignatelli P, Gaetano G & Violi F (2011). Effects of resveratrol and other wine polyphenols on vascular function. *J. of Nutr. Biochem.*, **22**:201-11.

Guchu E, Díaz-Maroto M C, Pérez-Coello M S, González-Viñas M A & Ibáñez M D C, (2006). Volatile composition and sensory characteristics of Chardonnay wines treated with American and Hungarian oak chips. *Food Chem.*, **99**: 350-359.

Guerrero-Beltran J A & Barbosa-Canovas G, (2004). Review: advantages and limitations on processing foods by UV light, *Food Sci. Technol. Int.*, **10**: 137- 147.

Gusman J, Malonne H & Atassi G, (2001). A reappraisal of the potential chemopreventive and chemotherapeutic properties of resveratrol. *Carcinogenesis*, **22**: 1-7.

Harding G, (2005). "A Wine Miscellany" Clarkson Potter Publishing, New York ISBN 0-307-34635-8, 5-9.

Heard G M & Fleet G H (1988). The effects of temperature and pH on the growth of yeast species during the fermentation of grape juice. *J. App. Bacteriol.*, **65**: 23–28.

Henschke P, (1997). Stuck fermentation – causes, prevention and cure. ASVO Seminar, 30-38.

Hernanz D, Gallo V, Recamales Á F, Meléndez-Martínez A J, González-Miret M L & Heredia F J, (2009). Effect of storage on the phenolic content, volatile composition and colour of white wines from the varieties Zalema and Colombard. *Food Chem.*, **113**: 530-537.

Hornsey S I, (2012). *Brewing*. Cambridge: *Royal Societ. Chem.*, **22**:1-2.

Hudina M & Štampar F, (2000). Sugars and organic acids contents of European (*Pyrus communis* L.) and Asian (*Pyrus serotina* Rehd.) pear cultivars. *Acta Aliment*, **29(3)**: 217-230.

Hu X S & Li J H, (1995). *Modern fruit and vegetable juice processing technology*, China Light Industry Press, Peking, 48-89.

Hui HY, Chan K & Khachatourians G G, (1994). *Food Biotechnology*. USA: Wiley-IEEE, 847-848.

Iketani H, Manabe T, Matsuta N, Akihama T & Hayashi T (1998). Incongruence between RFLPs of chloroplast DNA and morphological classification in east Asian pear (*Pyrus* spp.). *Gen. Resis. Crop Evol.*, **45**: 533–539.

Iland P, Ewart A, Sitters J, Markides A & Bruer N, (2000). *Techniques for Chemical Analysis and Quality Monitoring During Winemaking*. Australia: Patrick Iland Wine Promotions, **1**:6-7.

IngledeW W M & Kunkee R, (1985). Factors influencing sluggish fermentations of grape juice. *Am. J. Enol. Vitic.*, **36**:65-76.

Jackson R S, (2000). *Principles, Wine Practice Science Perception*. California, USA: Academic Press, 283-427.

Jiranek V, Langridge P & Henschke P A, (1995). Amino acid and ammonium utilisation by *Saccharomyces cerevisiae* wine yeasts from a chemically defined medium. *Am. J. Enol. Vitic.*, **46**: 75-83

Jones R P, Pamment N & Greenfield P F, (1981). Alcohol fermentation by yeasts; the effect of environmental and other variables, *Process Biochem.* :16-42.

Kader A A, Kasmire R F, Mitchell F G, Reid M S, Sommer N F & Thompson J F, (1985). *Postharvest technology of horticultural crops*. Cooperative Extension, University of California. Special Publication, 3311: Pp 192. MoAD. 2014. Statistical information on Nepalese agriculture 2013/2014 (2070/071). Government (different years), Ministry of Agriculture Development, Singhdurbar, Kathmandu.

Kallio H, Hakala M, Pelkkikangas A M & Lapveteläinen A, (2000). Sugars and acids of strawberry varieties. *Eur. Food Res. Technol.*, **212**: 81- 85.

Karki T, (1986). Some Nepalese Fermented Foods and Beverages. In *Traditional Foods: some products and technologies*. Mysore: *Cen. Food Technol. Res. Insti.*

Karthikeyan M, Kanchana D, Saranraj P & Karunya SK (2015). Quantification of metabolic products from lactic acid bacteria isolated from traditional fermented dairy products. *Afri. Edu. Indic.*, **8**: 1-15.

Killian R E & Ough C S, (1979). Fermentation ester-formation and retention as affected by fermentation temperature. *Am. J. Enol. Vitic.*, **30**:301-305.

Knorr D, Geulen M, Grahl T & Sitzmann W, (1994). Food application of high electric Field pulses, *Trends in Food Sci. Technol.* **5**: 71-75.

Koussissi E, Dourtoglou V G, Ageloussis G, Paraskevopoulos Y, Dourtoglou T, Paterson A & Chatzilazarou A, (2009). Influence of toasting of oak chips on red wine maturation from sensory and gas chromatographic headspace analysis. *Food Chem.*, **114**:1503-1509.

Ku C S & Mun S P, (2008). Optimization of the extraction of anthocyanin from Bokbunja (*Rubus coreanus* Miq.) marc produced during traditional wine processing and characterization of the extracts. *Biores. Technol.*, **99**: 8325–8330.

Kuboye A O, Oniwinde A B & Akinrele I A, (1978). Production of Alcoholic Beverages from Ripe Pineapples, Plantain, and Bananas, **Vol. 2**, Pp.78-80. *Nigerian Insti. Food Sci. Technol.* Lagos, Nigeria.

Kumar D P, Jayanthi M, Saranraj P & Karunya S K (2015). Effect of potassium sorbate on the inhibition of growth of fungi isolated from spoiled Bakery products. *Life Science Arch*, **1(2)**:17-22.

Lacroux F, Tregat O, Van Leeuwen C A, Tominaga T, Lavigne- Cruège V & Dubordieu D, (2008). Effect of foliar nitrogen and sulphur application on aromatic of *Vitis vinifera* L. cv. sauvignon blanc. *Int. J. Sci. Vin.*, **42**:75.

Lee P R, Chong I S, Yu B, Curran P & Liu S Q, (2012). Effect of sequentially inoculated *Williopsis saturnus* and *Saccharomyces cerevisiae* on volatile profiles of papaya wine. *Food Res. Insti.*, **45(1)**:77-83.

Leroy M J, Charpentier M, Duteurtre B, Feuillat M & Charpentier C, (1990). Yeast autolysis during Champagne aging. *Am. J. Enol. Vitic.*, **41**: 21-28.

- Liberatore M T, Pati S, Nobile M A D & Notte E, (2010). Aroma quality improvement of Chardonnay white wine by fermentation and ageing in barrique on lees. *Food Res. Insti.*, **43**: 996-1002.
- Li X, Zhang J Y, Gao W Y, Wang Y, Wang H Y, Cao J G & Huang L Q, (2012). Chemical composition and anti-inflammatory and antioxidant activities of eight pear cultivars. *J. Agric. Food Chem.*, **60**:8737–8744.
- Madigan M T, Martinko J M & Parker J, (2013). *Brock Biology of Microorganisms*. 10th ed. Prentice Hall, London: Pearson Education Inc.
- Magasanik B & Kaiser C A, (2002). Nitrogen regulation in *Saccharomyces cerevisiae*. *Gene* **290**: 1- 18.
- Manginot C, Roustan J L & Sablayrolles J M, (1998). Nitrogen demand of different yeast strains during alcoholic fermentation. Importance of the stationary phase. *Enz. Micro. Technol.*, **23**: 511- 517.
- McGovern P E, (2009). *Uncorking the Past: The Quest for Wine, Beer, and Other Alcoholic Beverages*. Berkeley: University of California Press.
- Meiselman H L & Schutz H G, (2003). History of food acceptance research in US Army. *Appetite*, **40**: 199–216.
- Mena P, Vilaplana A G, Marti N & Viguera C G, (2012). Pomegranate varietal wines: Phytochemical composition and quality parameters. *Food Chemistry*, **133**: 108-15.
- Mengzhen M A, QIAN Zilin, Luting W U & JIANG Heti, (2014). Research of the brewing technology of semi-dry crisp pear wine. College of Food Science, Southwest University. Research article, science and technology of food industry.
- Mohanty S, Ray P, Swain M R & Ray R C, (2006). Fermentation of cashew (*Anacardium occidentale* L.) apple into wine. *J. Food Proc. Preserv.*, **30(3)**:14-22.
- Monteiro F F & Bisson L F, (1991). Biological assay of nitrogen content of grape juice and prediction of sluggish fermentations. *Am J. Enol. Vitic.*, **42**: 44-57.
- Montgomery M W & Petropakis H J, (1980). Inactivation of Bartlett Pear Polyphenol Oxidase with Heat in the Presence of Ascorbic Acid. *J. Food Sci.*, **45**: 1090.
- Moreno N J & Azpilicueta C A, (2007). Binding of oak volatile compounds by wine lees during simulation of wine ageing. *LWT - Food Sci. Technol.*, **40**: 619-624.

Morrot G, (1999). Can we improve taster performance? In Winetasting, special issue, *J. Int. des Sci. de la Vigne et du Vin*, 29–35.

Moyer J C & Aitken H C, (1980). Apple juice. In P. E. Nelson & D. K. Tressler (Eds.), *Fruit and vegetable juice proc. Technol.*, 212-267.

Nevarés I, Alamo M D & Gonzalez-Muñoz C, (2010). Dissolved oxygen distribution during micro-oxygenation. Determination of representative measurement points in hydroalcoholic solution and wines. *Analytica Chimica Acta*, **660**: 232-239.

Ngadi M O, Smith J P & Cayouette B, (2003). Kinetics of ultraviolet light inactivation of *Escherichia coli* O157:H7 in liquid foods. *J. Sci. Food Agric.*, **83**: 1551-1555.

Niu Lili, Zhang Shenggui, Bi Yang & Han Shunyu, (2016). Study on the Brewing Technology of Pear Wine (College of Food Science and Engineering, Gansu Agricultural University, Lanzhou, Gansu 730070, China).

Noble A C, (ed.), (2002), *Astringency and Bitterness of Flavonoid Phenols*, Washington, DC, *Am. Chem. Socie.*.

Noll R G (2017). The wines of West Africa: History, technology and tasting notes. *J. Wine Eco.*, **3**: 85-94.

NSW Primary Industries (2002). *Nashi Asian pear varieties*, nitaka. Agfact H4.1.14

Obaedo M E & Ikenebomeh M J, (2015). Microbiology and production of banana (*Musa sapientum*) wine. *Nige. J. Microbiol.*, **23(18)**: 86-91.

Ough C S, Huang Z, An D & Stevens D, (1991). Amino acid uptake by four commercial yeasts at two different temperatures of growth and fermentation. Effects of growth and fermentation. Effects on urea excretion and reabsorption. *Am. J. Enol. Vitic.*, **42**; 26-40.

Palacios S, Vasserot Y & Maujean A, (1997). Evidence for sulfur volatile products adsorption by yeast lees. *Am. J. Enol. Vitic.*, **48**; 525-526.

Patricia R R, Carmen G B, Raquel R O, Beatriz C G & Jesús S G, (2013). Nutrition and Bromatology Group, Department of Analytical and Food Chemistry; Food Science and Technology Faculty, University of Vigo—Ourense Campus, E-32004, Ourense.

Perez-Nevado F, Albergaria H, Hogg T & Girio F (2006). Cellular death of two non-*Saccharomyces* wine-related yeasts during mixed fermentation with *Saccharomyces cerevisiae*. *Int. J. Food Microbiol.*, **108**: 336–345.

Petropakis H J & Montgomery M W, (1984). Improvement of Colour Stability of Pear Juice Concentrate by Heat Treatment. *J. Food Technol.*, **19**: 91.

Pina C, Santos C, Couto J A & Hogg T (2004). Ethanol tolerance of five non-Saccharomyces wine yeasts in comparison with a strain of *Saccharomyces cerevisiae*-influence of different culture conditions. *Food Microbiol.*, **21**: 439–447.

Petit C & Sieffermann J M, (2007). Testing consumer preference for iced-coffee: Does the drinking environment have any influence. *Food Quality and Preference*, **18**:161–172.

Pozo-Bayón M A, Martínez-Rodríguez A, Pueyo E & Moreno-Arribas M V, (2009). Chemical and biochemical features involved in sparkling wine production: from a traditional to an improved winemaking technology. *Trends in Food Sci. & Technol.*, **20**; 289-299.

Pretorius I S, Van der Westhuizen T J & Augustyn O H, (1999). Yeast biodiversity in vineyards and wineries and its importance to the South African wine industry. *S. Afr. J. Enol. Vitic.*, **20**: 61±74.

Ranken M D, Kill R C & Baker C G, (1997). *Food Industries Manual*. UK: Blackie Academic and Professional.

Requena T, Monagas M, Bayo M P, Martin P J, Bartolome B & Campo R, (2015). Perspectives of the potential implications of wine polyphenols on human oral and gut microbiota. *Trends Food Sci. Technol.*, **21**:332-44.

Ribéreau-Gayon P, Dubourdieu D, Donèch B, & Lonvaud A, (1998). *Handbook of enology* (vol.1). John Wiley & Sons Ltd. Paris.

Robinson J, (2016). *The Oxford Companion to Wine*. 3rd ed. USA: Oxford University Press, **7**: 79-87.

Rodríguez-Bencomo J J, Ortega-Heras M, Pérez-Magariño S & González-Huerta C, (2009). Volatile compounds of red wines macerated with Spanish, American, and French oak chips. *J. Agric. Food Chem.*, **57**: 6383-6391.

Sablayrolles J M, (1996). Sluggish and stuck fermentations. Effectiveness of ammonium nitrogen and oxygen additions. *Vitic. Enol. Sci.*, **51**; 147-151.

Sakuma T, (1995). Brief introduction to the activities of Horticulture Development Project – II. Proceeding of the Seminar on Fruit Development in Nepal, 22- 24 February 1995. Horticulture Development project, Kirtipur and Department of Agriculture Development, Harihar Bhawan, Lalitpur. 155-162.

San Martín M F, Barbosa-Cánovas G V & Swanson B G, (2002). Food processing by high hydrostatic pressure. *Crit. Rev. Food Sci. Nutr.*, **42(6)**: 627-645.

Sanza M D & Domínguez I N, (2006). Wine aging in bottle from artificial systems (staves and chips) and oak woods Anthocyanin composition. *Analytica Chimica Acta*, **563**: 255-263.

Sarah J L, Mesnidrey L, Maguerite E & Biosseau M. 2017 Laboratory screening of pineapple germplasm for resistance to the lesion nematode *Pratylenchus brachyurus*. *Acta Horti*; **425**:179-86.

Saranraj P, Naidu M A & Sivasakthivelan P. 2013 Lactic acid bacteria and its antimicrobial properties: A review. *Int. J. Pharma Biol. Arch*, **4(11)**: 24-33.

Sarkar D, Chandrakant Ankolekar, Marcia Pinto & Shetty K, (2014): Dietary functional benefits of Bartlett and Starkrimson pears for potential management of hyperglycemia, hypertension and ulcer bacteria *Helicobacter pylori* while supporting beneficial probiotic bacterial response: *Food Res. Int.*

Sarni F, Moutounet M, Puech J L & Rabier P, (1990). Effect of heat treatment of oak wood extractable compounds. *Holzforschung*. **44**; 461-466.

Segade R, Vázquez S, Rodríguez V & Martínez R. (2009). Influence of training system on chromatic characteristics and phenolic composition in red wines. *Eur. Food Res. Technol.*, **229**: 763-770.

Sha Shoufeng, Li Juncai, Jun Wu & Zhang Shaoling, 2011. Characteristics of organic acids in the fruit of different pear species. *Afr. J. Agric. Res.*, **Vol. 6(10)**, 2403-2410.

Shahidi F (2009). Nutraceuticals and functional foods: Whole versus processed foods. *Trends in Food Sci. Technol.*, **20**:376-87.

Shrestha H & Karki T, (1999). Fermentation Process of Nepal Murcha Starters, Abstract Presented at the National Science Conference Organized by Royal Nepal Academy of Science and Technology, Kathmandu, Nepal.

Sivasakthivelan P, Saranraj P & Sivasakthi S, (2014). Production of bioethanol by *Zymomonas mobilis* and *Saccharomyces cerevisiae* using sunflower head wastes - A comparative study. *Int. J. Microbiol. Res.*, **5(208)**:16.

Slinkard K & Singleton V L, (1997). Total Phenol Analysis: Automation and Comparison with Manual Methods. *Am. J. Enol. Vitic.*, **28**:49-55

Steinkraus K H, (1992). Lactic acid fermentations. In: Applications of Biotechnology to Traditional Fermented Foods. Washington D.C, USA: *Report of an Ad Hoc Panel of the Board on Sci. Technol. Int. Develop., National Academy Press.*

Tatum J H, Shaw P E & Berry R E, (1969). Degradation Products from Ascorbic Acid. *J. of Agri. and Food Chem.*, 17-38.

Thomas D S & Rase A H, (1979). Inhibitory effect of ethanol on growth and solute accumulation by *Saccharomyces cerevisiae* as affected by plasma-membrane lipid composition, *Arch. Microbiol.*, **122**:49.

Trappey A F, Johnson C E & Wilson P W, (2008). Comparison of Juice Extraction Methods from Fresh and Frozen Mayhaw (*Crataegus opaca* Hook). *Fruit, Int. J. of Fruit Sci.*, **7(3)**:41-64.

Tuorila H & Monteleone E, (2009). Sensory food science in the changing society: Opportunities, needs and challenges. *Trends Food Sci. Technol.*; **20**:54-62.

Van Rooyen P C & Tromp A, (1982). The effect of fermentation time (as induced by fermentation and must conditions) on the chemical profile and quality of chinin blanc wine. *S. Afr. J. Enol. Vitic.* ; **3**:75.

Viegas C A, Sa-Correia I & Navais J M, (1986). Synergistic inhibition of the growth of *Saccharomyces buyanus*, *Biotechnol. and Bioeng.* , **28**:761.

WANG Han-ping, (2012). Study on a Kind of Brewing Technology of the Full-fermentation Complex Fruit Wine. (Department of Life Sciences, Shaanxi Institute of Education, Xian, Shaanxi 710061)

Wang J, Xu J Z, Chen H J, Zhang H J & Li S L, (2002). The determination of volatile matters and amino acids content in the fruit of Ya pear. *Food Technol.*, **9**:71–73.

Waterhouse A L (1999). Determination of Total Phenolics, in Current Protocols in Food Analytical Chemistry, I1.1.1-I1.1.8, Wrolstad, R.E., Wiley, 2001, or Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu Reagent. *Methods in Enzymol.*, **299**; 152-178.

Wilson C A, (1993). Liquid Nourishment: Potable foods and stimulating drinks, Edinburgh University Press, **p**; 94

Wimal Siri P, Sinnatamby A, Samaranayake S & Samarasinghe C R, (1971). Cashew Apple Wine. Sri Lanka: Industry Prospect Report, Ind. Develop. Board, **p**.44.

XIE Ding, ZHONG Hai-yan, CUI Tao, ZHANG Zhao-fei, YUAN Lie-jiang & HU Xiao-yi, (2014). Analysis of Aroma Components in Sand Pear Juice by Solid-phase Micro-extraction Coupled with Gas Chromatography-mass Spectrometry.

Yokotsuka T, (2015). Fermented protein foods in the orient, with emphasis on shoyu and miso in Japan. In: Wood BJ, editor. Microbiology of Fermented Foods. UK: Elsevier Applied Science Publishers.

Zárate-Rodríguez E, Ortega-Rivas E, & Barbosa-Cánovas G.V, (2001). Effect of membrane pore size on quality of ultrafiltered apple juice. *Int. J. Food Sci. Tech.*, **36(6)**: 663-667.

Zott K, Miot-Sertier C, Claisse O, Lonvaud-Funel A & Masneuf- Pomarede I, (2008). Dynamics and diversity of non- *Saccharomyces* yeasts during the early stages in winemaking. *Int. J. of Food Microbiol.* **125**:197–203.

## APPENDIX

YEPD Agar	(Ingredients g/L)
Yeast extracts	3g
Peptone	5g
Glucose	10g
Agar	20g
Distilled water	1000ml

Table: 9-point hedonic scale with score and their corresponding labels.

Score	Remarks
9	Like extremely
8	Like very much
7	Like moderately
6	Like slightly
5	Neither like nor dislike
4	Dislike slightly
3	Dislike moderately
2	Dislike very much
1	Dislike extremely

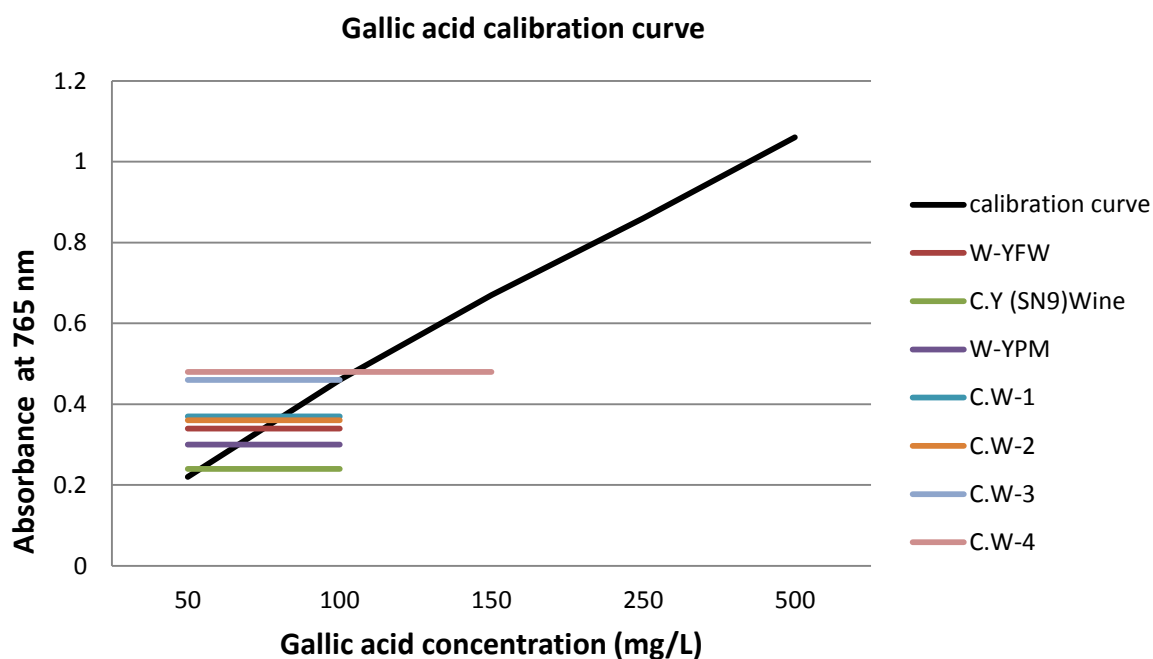
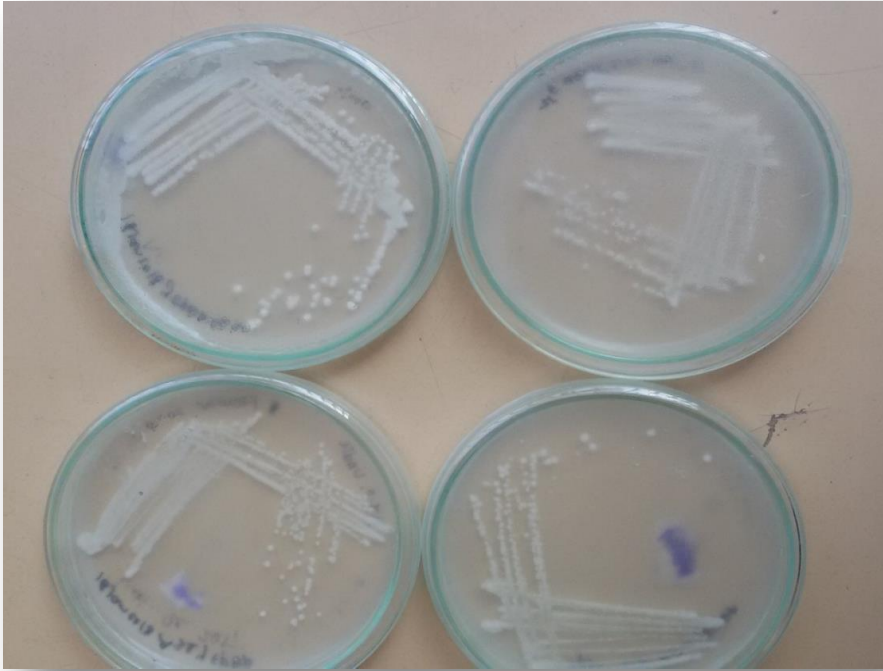
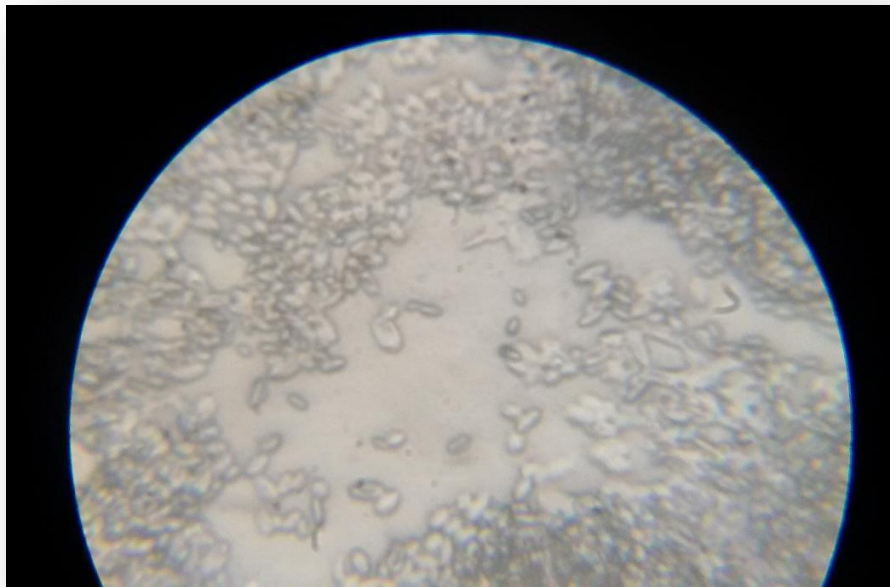


Fig: Calibration curve of gallic acid at wavelength of 765nm for total phenol determination.

## PHOTO GALLERY



**Fig:** Colonies of yeast appeared on YEPDA plates from different sample



**Fig:** Microscopic observation of yeast cell in 40X



**Fig:** Pilot scale wine production using pear



**Fig:** Sensory evaluation



**Fig:** Sensory evaluation



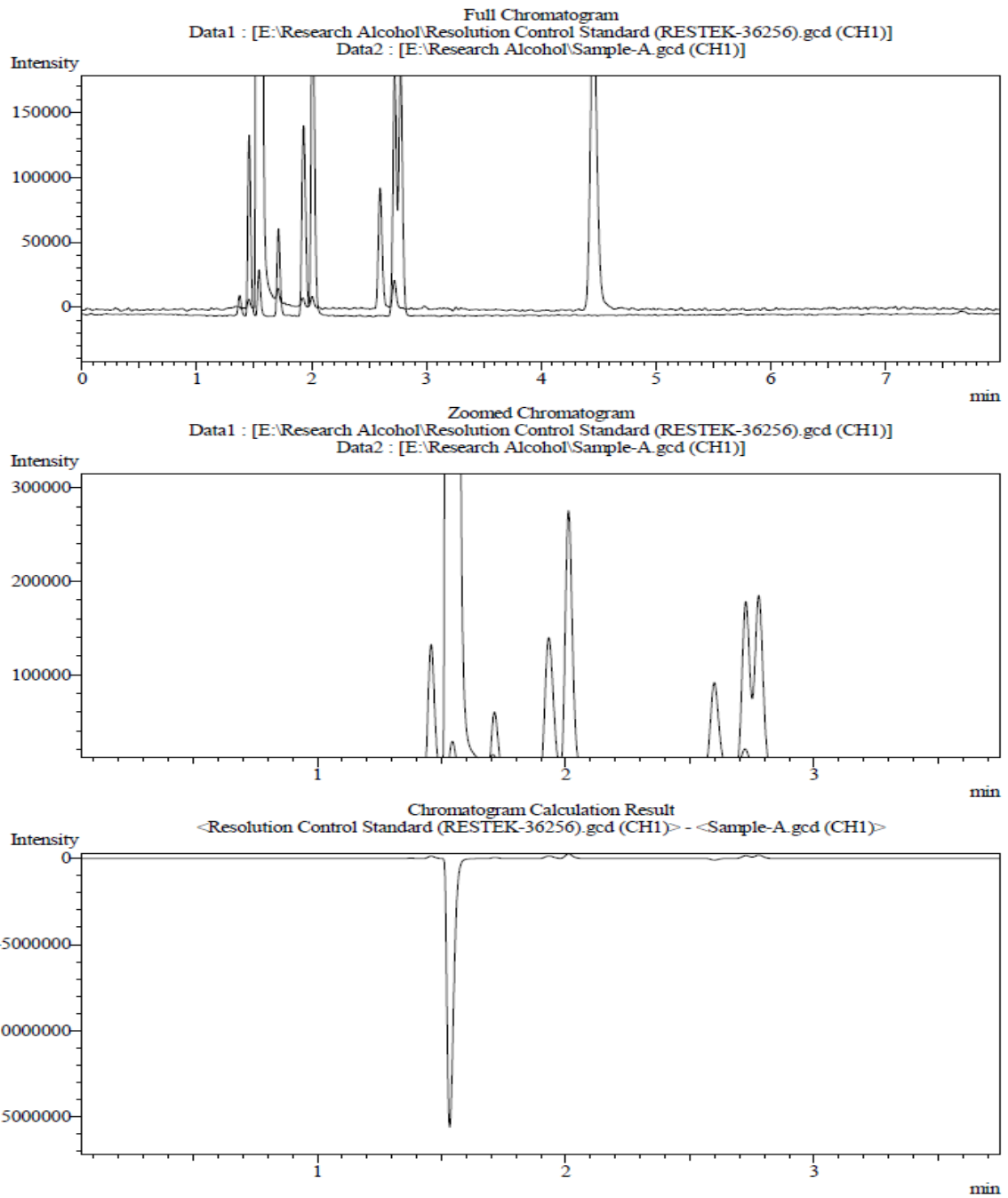
**Fig:** Sensory testing room



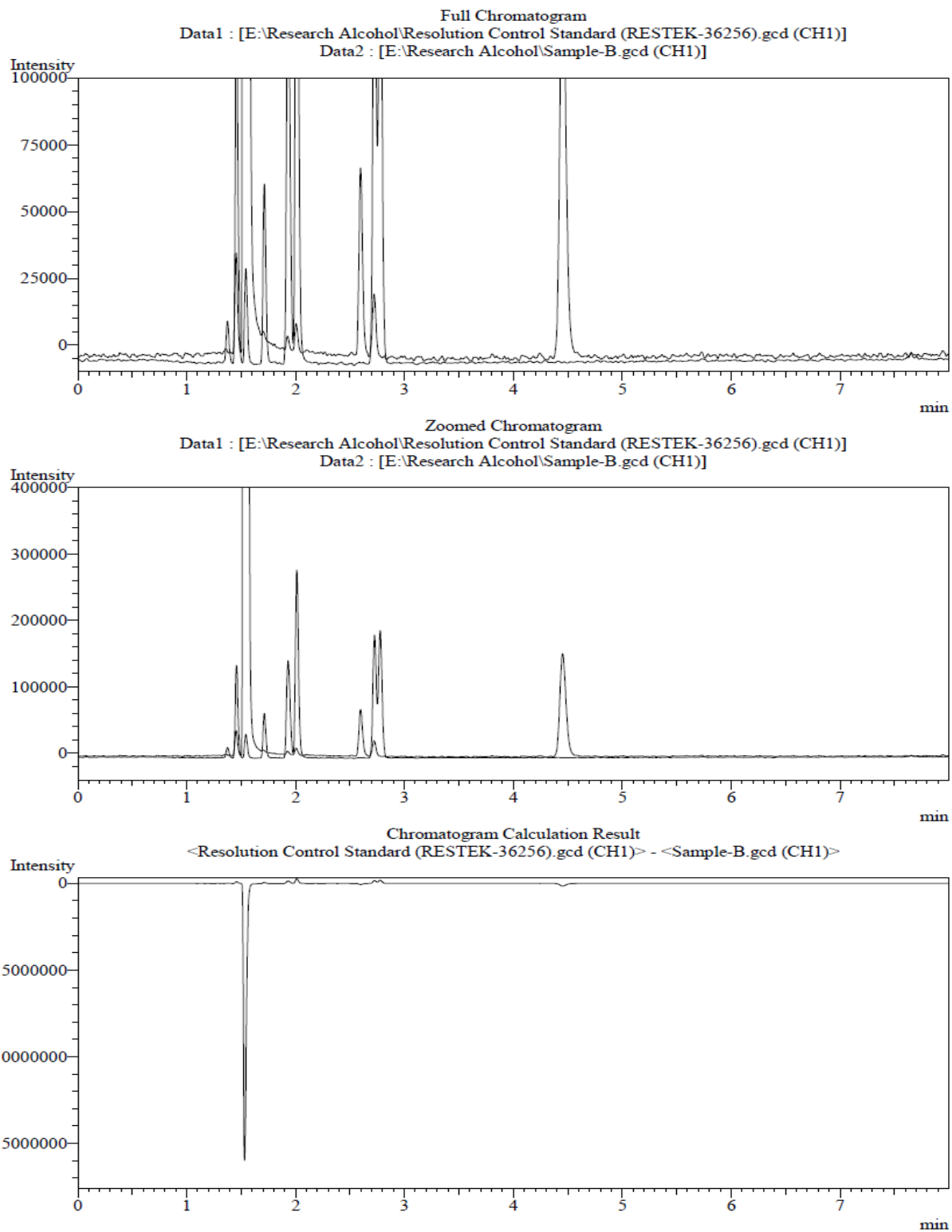
**Fig:** Sensory test performed by Dr. Ganga P. Kharel PhD



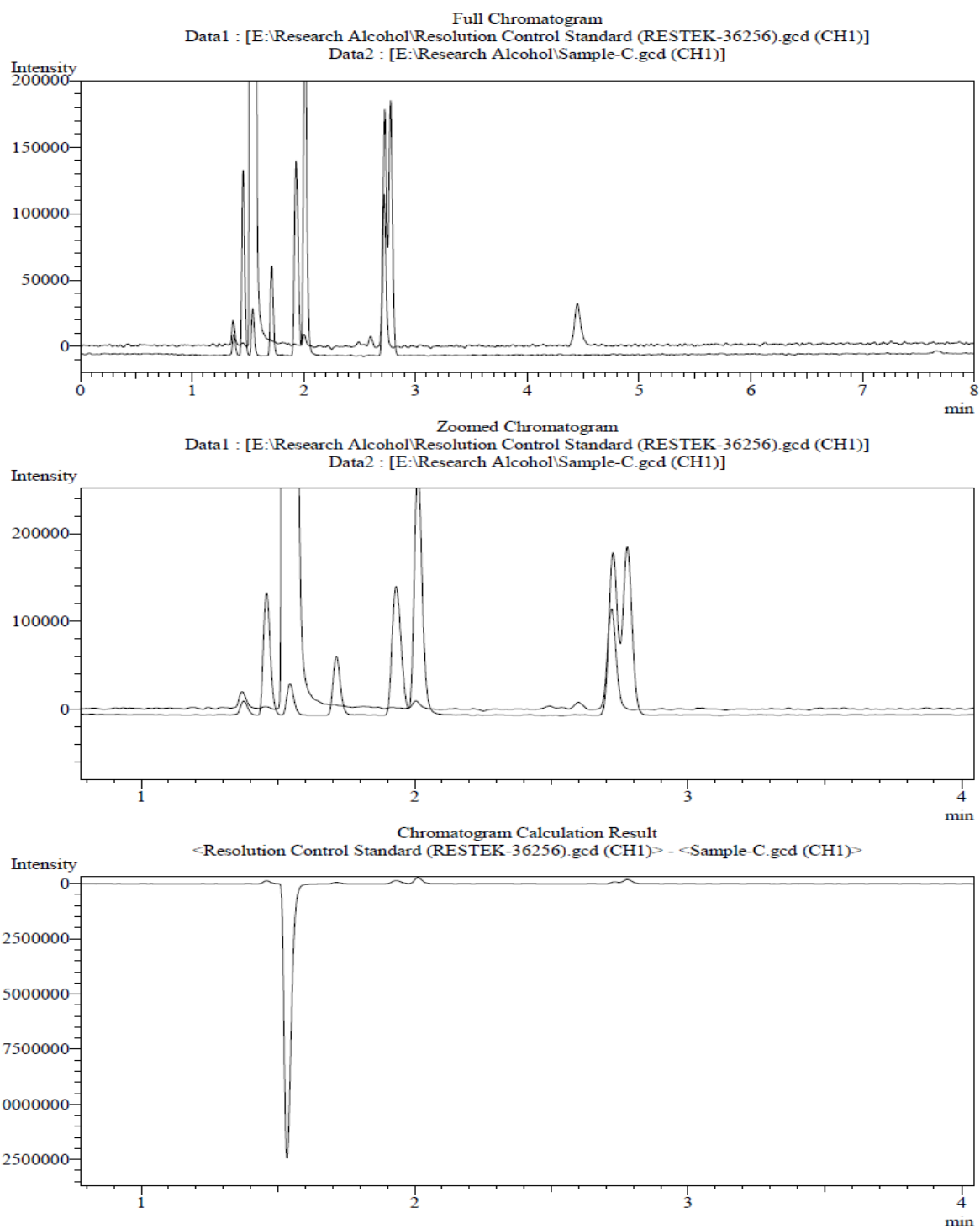
**Fig:** Sensory test performed by Dr. Krishna D. Manandhar PhD



**Fig:** Chromatogram of standard volatile mix.

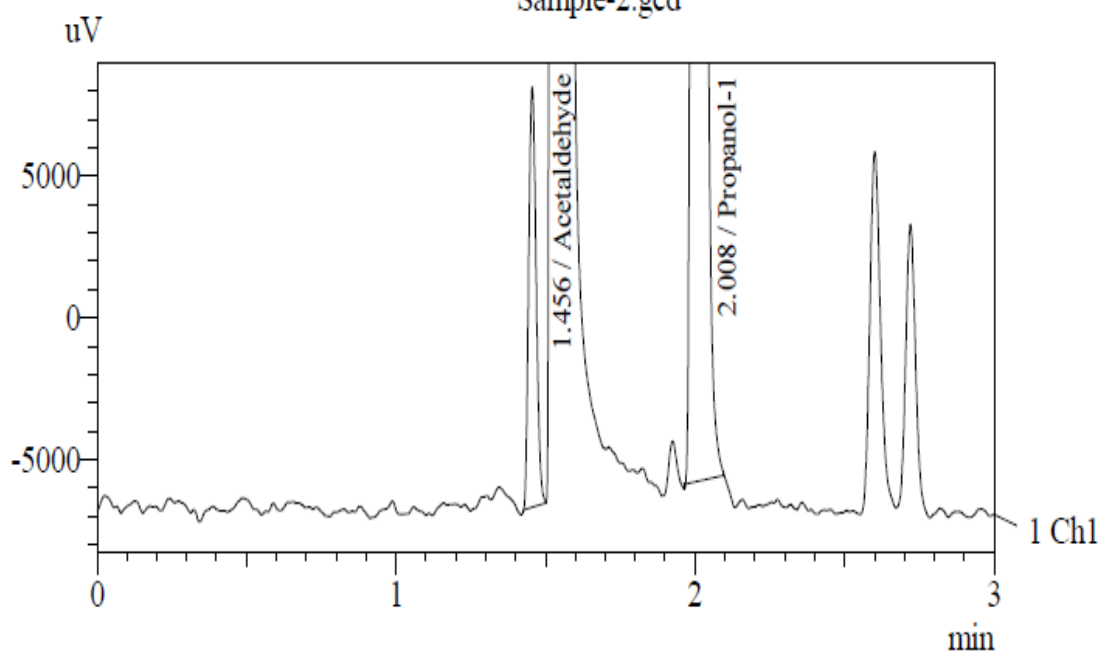


**Fig:** Chromatogram of wine volatile fermented by the yeast isolated from Palpa's murcha



**Fig:** Chromatogram of commercial wine

Summary(Compound)  
Sample-2.gcd



<< Channel 1 >>

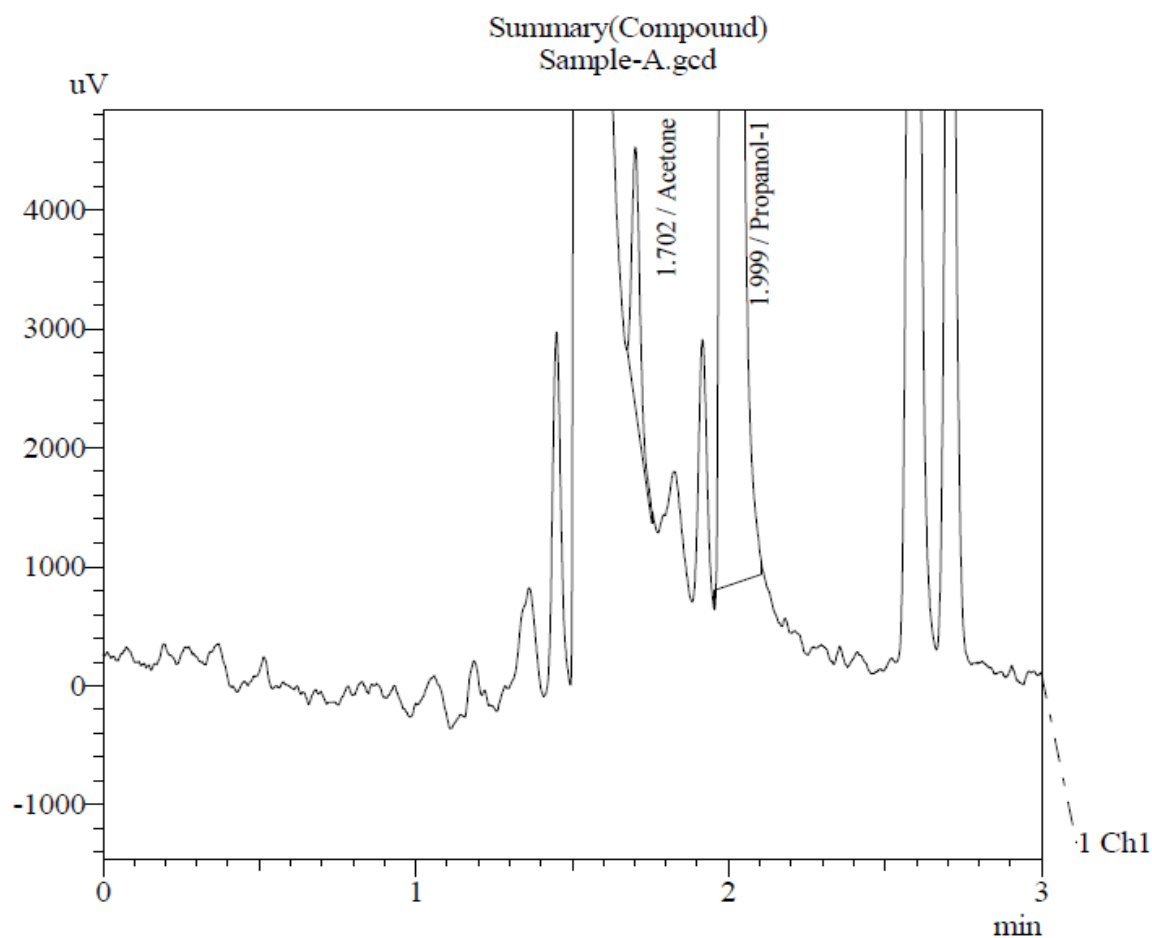
ID#1 Compound Name: Acetaldehyde

Title	Ret. Time	Conc.	Units
Sample-2.gcd	1.456	41.271	mg/dl

ID#2 Compound Name: Propanol-1

Title	Ret. Time	Conc.	Units
Sample-2.gcd	2.008	0.000	mg/dl

Fig: Chromatogram of commercial wine



<< Channel 1 >>

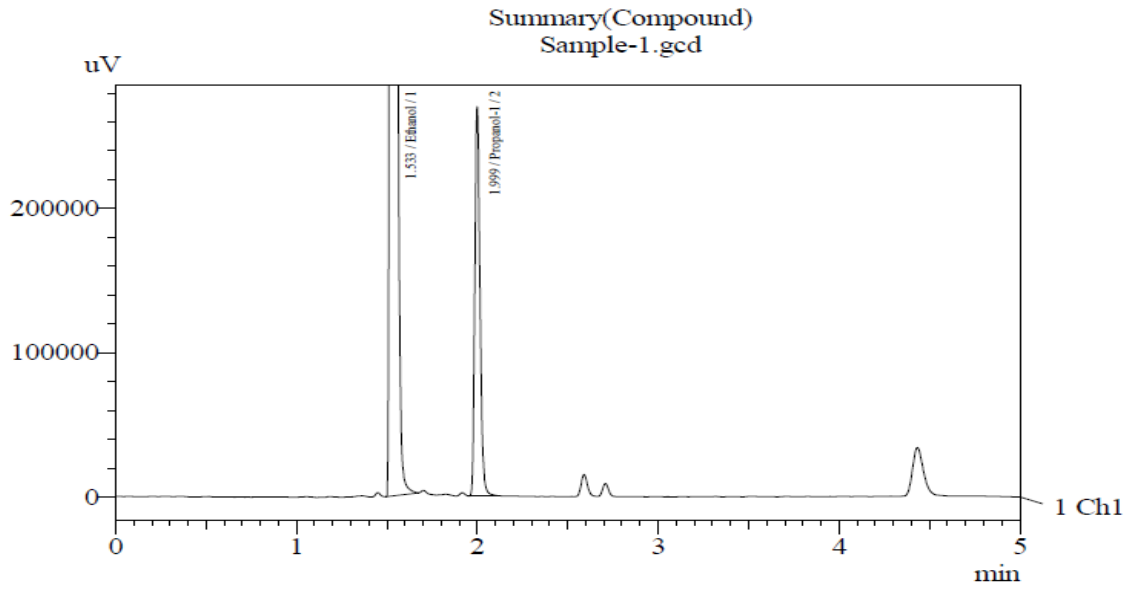
ID#1 Compound Name: Acetone

Title	Ret. Time	Conc.	Units
Sample-A.gcd	1.702	3.169	mg/dl

ID#2 Compound Name: Propanol-1

Title	Ret. Time	Conc.	Units
Sample-A.gcd	1.999	0.000	mg/dl

**Fig:** Chromatogram of wine volatile fermented by the yeast isolated from Palpa's mucha



<< Channel 1 >>

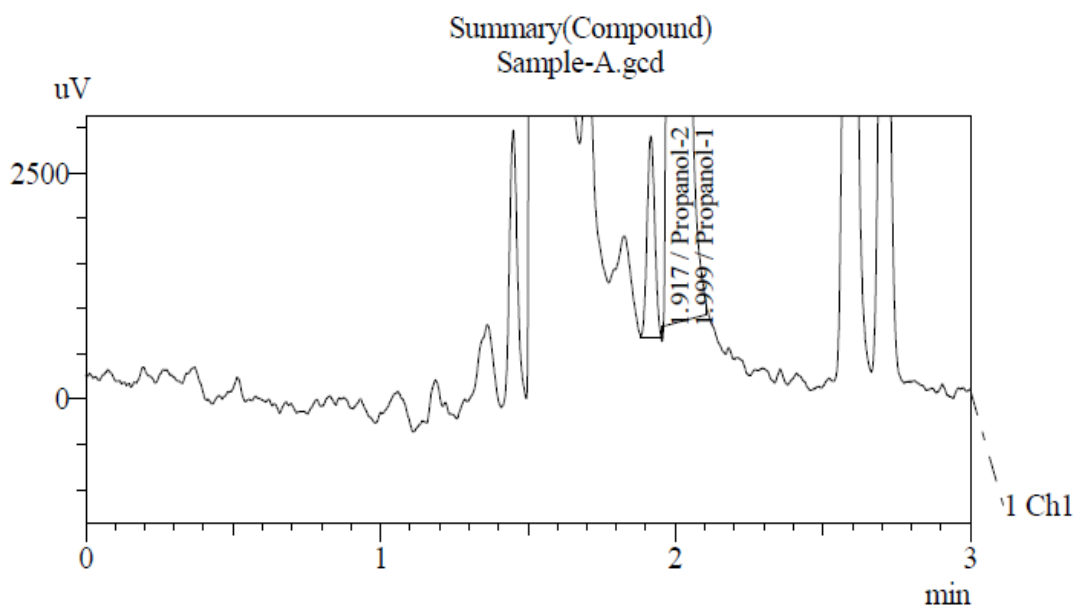
ID#1 Compound Name: Ethanol

Title	Ret. Time	Conc.	Units
Sample-1.gcd	1.533	11093.109	mg/dl

ID#2 Compound Name: Propanol-1

Title	Ret. Time	Conc.	Units
Sample-1.gcd	1.999	0.000	mg/dl

**Fig:** Chromatogram of wine volatile fermented by the yeast isolated from Palpa's mucha



<< Channel 1 >>

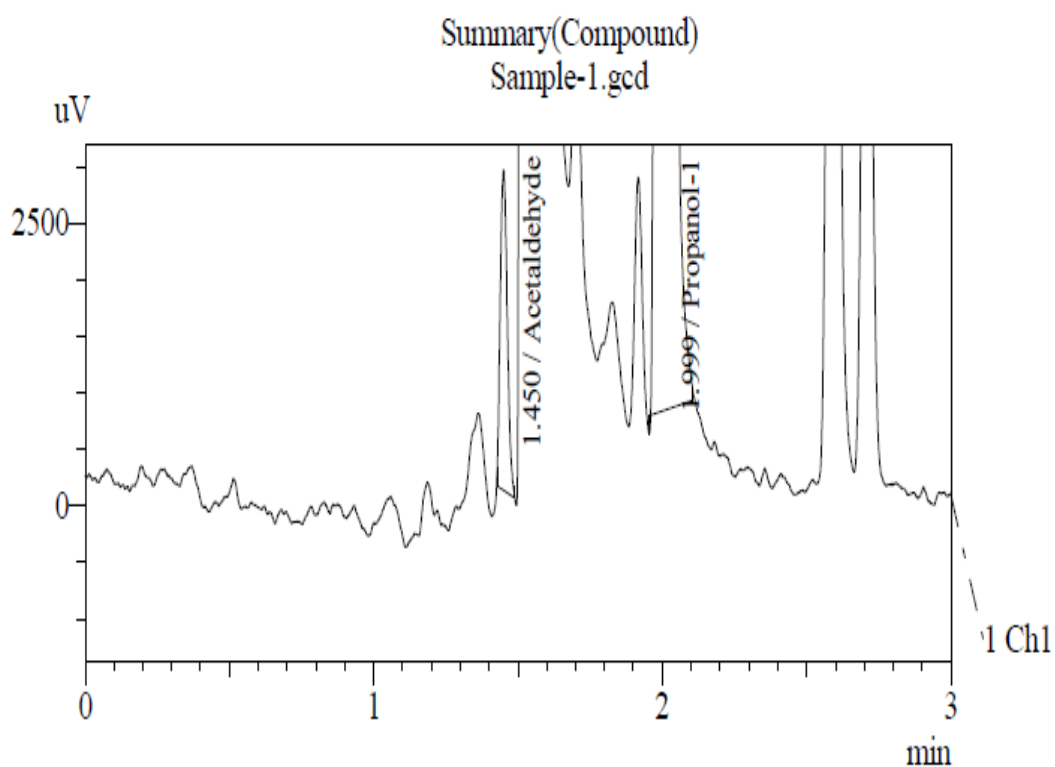
ID#1 Compound Name: Propanol-2

Title	Ret. Time	Conc.	Units
Sample-A.gcd	1.917	3.285	mg/dl

ID#2 Compound Name: Propanol-1

Title	Ret. Time	Conc.	Units
Sample-A.gcd	1.999	0.000	mg/dl

**Fig:** Chromatogram of wine volatile fermented by the yeast isolated from Palpa's mucha



<< Channel 1 >>

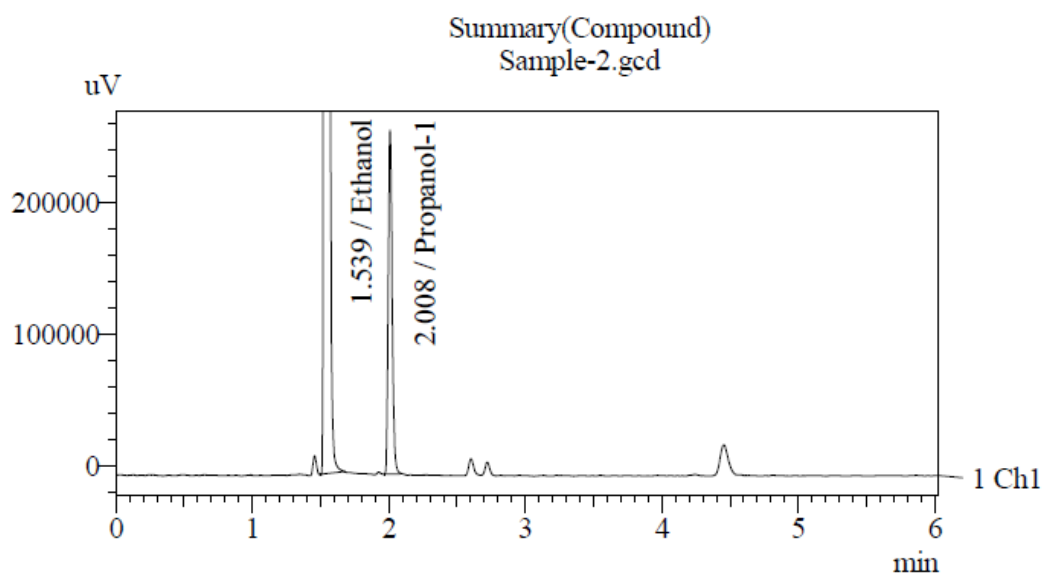
ID#1 Compound Name: Acetaldehyde

Title	Ret. Time	Conc.	Units
Sample-1.gcd	1.450	4.622	mg/dl

ID#2 Compound Name: Propanol-1

Title	Ret. Time	Conc.	Units
Sample-1.gcd	1.999	0.000	mg/dl

**Fig:** Chromatogram of wine volatile fermented by the yeast isolated from Palpa's mucha



<< Channel 1 >>

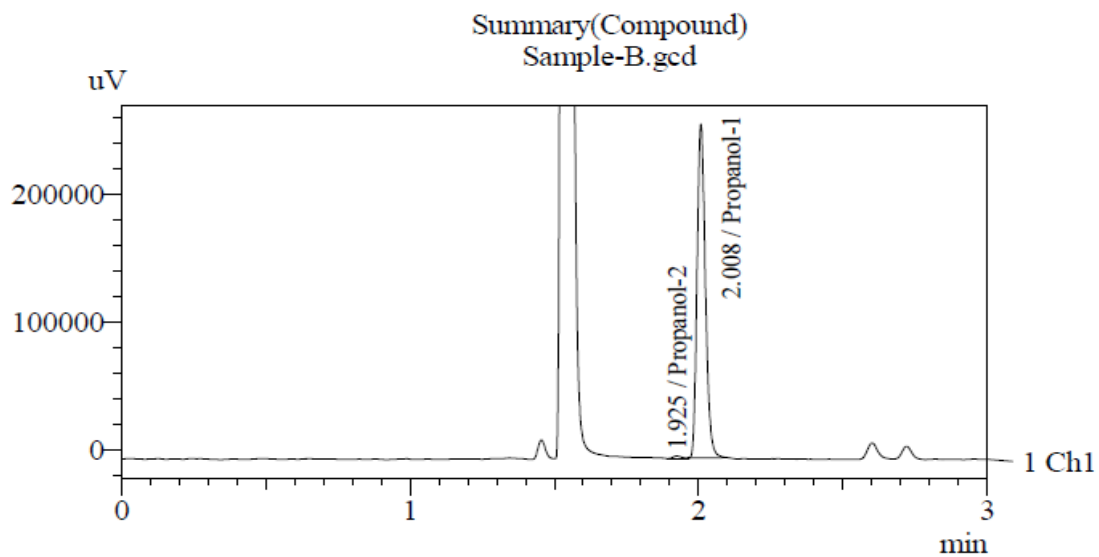
ID#1 Compound Name: Ethanol

Title	Ret. Time	Conc.	Units
Sample-2.gcd	1.539	12315.737	mg/dl

ID#2 Compound Name: Propanol-1

Title	Ret. Time	Conc.	Units
Sample-2.gcd	2.008	0.000	mg/dl

**Fig:** Chromatogram of wine fermented by yeast isolated from flower (*Argyrea nervosa*)



<< Channel 1 >>

ID#1 Compound Name: Propanol-2

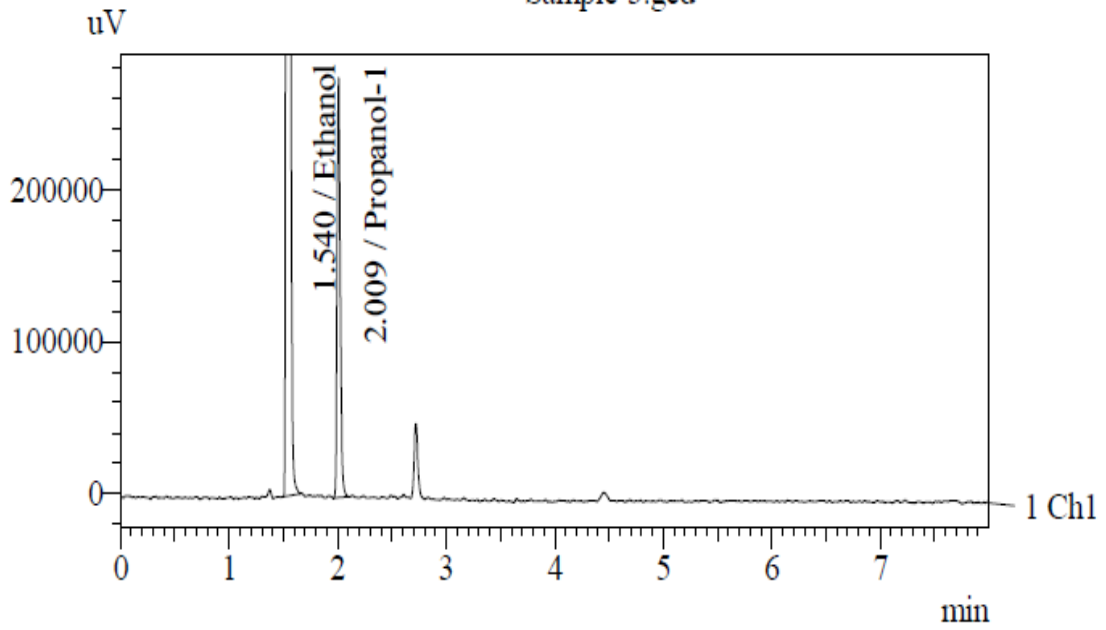
Title	Ret. Time	Conc.	Units
Sample-B.gcd	1.925	1.993	mg/dl

ID#2 Compound Name: Propanol-1

Title	Ret. Time	Conc.	Units
Sample-B.gcd	2.008	0.000	mg/dl

**Fig:** Chromatogram of wine fermented by yeast isolated from flower (*Argyrea nervosa*)

Summary(Compound)  
Sample-3.gcd



<< Channel 1 >>

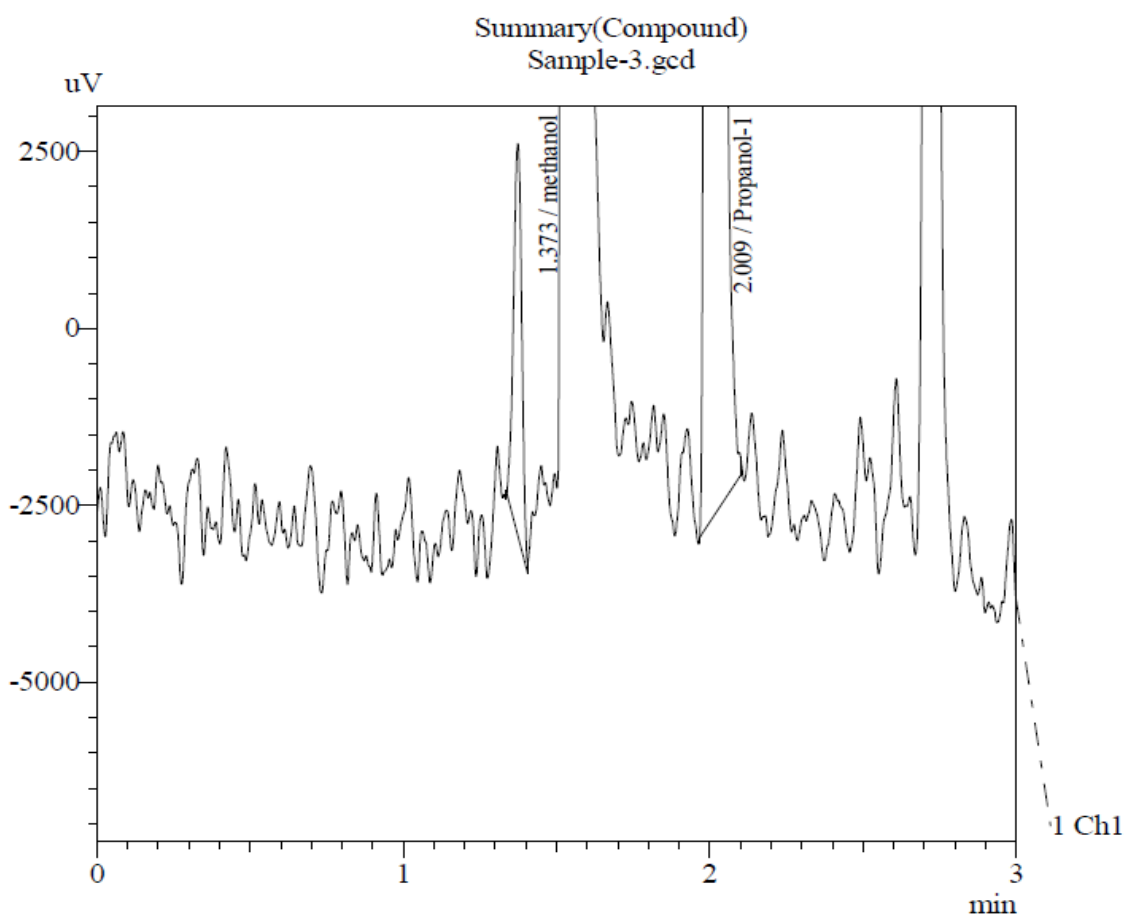
ID#1 Compound Name: Ethanol

Title	Ret. Time	Conc.	Units
Sample-3.gcd	1.540	9304.235	mg/dl

ID#2 Compound Name: Propanol-1

Title	Ret. Time	Conc.	Units
Sample-3.gcd	2.009	0.000	mg/dl

**Fig:** Chromatogram of commercial wine



<< Channel 1 >>

ID#1 Compound Name: methanol

Title	Ret. Time	Conc.	Units
Sample-3.gcd	1.373	13.147	mg/dl

ID#2 Compound Name: Propanol-1

Title	Ret. Time	Conc.	Units
Sample-3.gcd	2.009	0.000	mg/dl

**Fig:** Chromatogram of commercial wine