STUDY OF FERMENTATION PROCESS OF RICE WINE PREPARED USING LOCAL RAW MATERIALS

M. Sc. Thesis

(2018)

Submitted to

Central Department of Biotechnology

Tribhuvan University

Kirtipur, Kathmandu, Nepal

For partial Fulfillment of Requirement for the

Master of Science in Biotechnology

By

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TU Regd. No.: 5-2-0037-0956-2011

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Tribhuvan University

Kirtipur, Kathmandu, Nepal

Date…………………….

Recommendation

This is to certify that the research work entitled "**STUDY OF FERMENTATION PROCESS OF RICE WINE PREPARED USING LOCAL RAW MATERIALS**" has been carried out by **Mr. Dinesh Olee** under my 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. I, hereby, recommend this thesis for final evaluation.

……………………………………

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DINESH OLEE

LIST OF ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
CFU	Colony Forming Unit
CDBT	Central Department of Biotechnology
DNA	Deoxyribonuleic acid
DNS	Dinitrosalicylic acid
EDTA	Ethylene Di-amine Tetra-Acetic acid
EtBr	Ethidium Bromide
LAB	Lactic acid bacteria
MCFA	Medium Chain Fatty Acid
NCBI	National center for Biotechnology Information
NFW	Nuclease Free Water
OD	Optical Density
PBSA	Peptone Beef extract Starch agar
PCR	Polymerase Chain Reaction
ppm	parts per million
psi	Pound per Square Inch
Rcf/g	Relative centrifugal force/ g-force
RNA	Ribonucleic Acid
RNase	Ribonuclease
RT	Room Temperature
t	tonne
TA	Titrable acidity
TAE	Tris- Glacial Acetic acid – EDTA
Tm	Melting temperature
Tris	Tris-(Hydroxymethyl)-aminmethane
TSS	Total soluble solids
YEPDA	Yeast extract Peptone Dextrose Agar

ABSTRACT

Rice wine is alcoholic beverage made by simultaneous saccharification and fermentation by using mold and yeast respectively. In Nepal, traditional starter culture locally known as *murcha* has been used for fermentating locally available raw materials such as millet, rice, wheat, etc. The quality of alcoholic beverage always varies due to lack of process standardization in term of culture and process. Here, an attempt was made to isolate and screen mold and yeast from the *murcha* collected from different districts of Nepal and used in production of rice wine. The performance of mold was tested for saccharifying capacity and yeast for sugar, alcohol, pH tolerances and alcohol production. Most potent yeast isolates were identified by molecular tool using 18s universal primer. Seven molds isolates from murcha *were* tested for saccharification by halo zone on starch media, microscopic observation, liquefication and DNS test. All yeast isolates were also compared with commercial yeast (*Saccharomyces Bayanus SN9*). The results showed that all yeast isolates reproduced by budding, used glucose and sucrose as carbohydrate source during fermentation except *murcha* from Ramechhap district. *Murcha* used glucose and sucrose and none of them used starch, all isolates showed positive result of urea hydrolysis test except *murcha* from Parbat and commercial yeast. All isolates exhibiting growth in yeast-peptone-dextrose broth containing 40% glucose, 15% alcohol and pH ranges from 3-5.5. Alcohol production test measured by hand refractometer in YPD broth showed that isolates changed ˚Brix from 20 to 4.5 within 2 weeks at 28˚C. Among eight isolates, *murcha* from Parbat sample showed better performance in term of alcohol production. Yeasts isolates from Parbat and Dolakha district murcha showed 99% identity with *Saccharomyces boulardii* and *Saccharomyces cerevisiae* respectively during BLAST. All mold isolates showed better growth in Peptone, Beef extract, Starch and Agar (PBSA) media. All mold isolates produced fermentable sugar in ranges of 299.16μg/ml to 417.08 μg/ml. Yeast and mold from Parbat district was used for fermentation of rice wine. During fermentation change in acidity, ˚Brix and pH respectively 1.0-2.6 g/L, 1-5 and 4.1-3.4. After pasteurized rice wine showed 5% abv, 1.5 g/L succinic acid, 0.27 g/L amino acid, 5.8 ˚Brix, 4.7 pH, 0.56 g/100 mL glucose and not detected methanol (g/100 L).

Key words: *Murcha*, saccharification, fermentation, tolerance tests, rice wine

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CHAPTER 1
INTRODUCTION

1.1 Overview of rice

Rice (*Oryza sativa*) is a native grain of Southeast Asia is one of the leading food crops of the world and is second only to wheat in terms of annual production for food use. It is the main staple food for about 60 percent of the world's population. Rice is predominantly an Asian crop, 95 per cent of it is being produced and consumed in the Southeast Asian countries extending from Indo-Pakistan sub-continent to Japan. Rice is one of top most important food crops in Nepal. It commonly grown in terai region. In, Nepal production of paddy in the year 2012, 2013, 2014, 2015, 2016 were approximately 4,460; 5,072; 4,504; 5,047; 5(000t) respectively (ricepedia.org/Nepal).

1.2 Fermentation of alcoholic beverages from rice

Starch is the major constituent of rice and makes up to 90 per cent of rice in dry weight. The starch finds its application in food, pharmaceutical, textile, paper industries etc. The value of rice is increased by converting it into different products like flaked rice (chiura), expanded rice, rice flour and alcoholic beverages. It is also processed for production of maltose, dextrose, glucose syrups etc (Tamang, 2015). One may consider the utilization of rice in biomass formation, saccharification, alcoholic and acetic acid fermentation. Several indigenous rice beer-like products are produced around the world such as *sujen,* which is a popular local rice beer of Assam (India) (Das, 2014).

Additionally, distilled rice-based beverages exist, such as *shochu* which is a popular alcoholic drink that is distilled from *sake* (Murooka and Yamshita, 2008). Rice is also widely used as brewing adjunct in the USA and in Japan after maize (Hussain, 2012). As an adjunct, rice is favoured by some brewers because of its lower lipid and protein contents as compared with those of corn grits. Broken rice, obtained as a by-product of the edible rice milling industry, or rice grist is generally used in brewing. Rice is characterized by a neutral aroma and flavour and, when converted efficiently to fermentable sugars, yields a clean tasting, light beer (Arendt & Zannini, 2013).

Fermented foods are those which are produced by the action of specific microorganism or enzymes on different substrate, resulting in an acceptable product for human consumption. Fermented non-alcoholic food products are generally nutritious (Karki, 2013). Use of microorganism in preparing a food from locally available plant and animal material has been practiced since prehistoric times. Several traditional fermented foods beverages are produced at household level. Fermented foods make up important contribution to human diet in many countries because fermentation is an inexpensive technology that preserve foods and provide nutritional value and sensory properties (Karki, 2013). Development of spontaneous food fermentation was primarily governed by climate condition, availability of typical raw material, socio-cultural ethos, and ethical preferences.

Traditional fermented beverages have strong ritual importance and deep-rooted in the cultural heritage of the various ethnic groups of people. *Jand* and *raksi* are essential in marriage ceremony of non-Brahmin Hindu Nepalese and Buddhist tribes (Karki, 2013).

Alcoholic beverages are traditionally consumed in East Asia, Southeast Asia, and South Asia. Cereal wine is made from the fermentation of cereal starch that has been converted to sugars. Microbes are the source of the enzymes that convert the starches to sugar. In traditional fermentation starter are used as the starter culture. The starters are mixed culture of wide type of microorganism. Different versions of this drink exist, and they are locally known by different names; for instance: *sake* in Japan, *jand* in Nepal, *makgeolli* or *takju* in Korea (Huang, 2000).

1.2.1 *Sake*

Sake (Japanese rice wine) is an alcoholic beverage made by fermenting rice that has been polished to remove the bran (Furukawa, 2011). Unlike wine, in which alcohol is produced by fermenting grapes sugar. *Sake* is produced by a brewing process similar to that of beer, in which starch is converted into sugars and then ferment into alcohol. The brewing process for *sake* differs from the process for beer. In beer conversion from starch to sugar and from sugar to alcohol occurs in two distinct steps. Whereas in *sake* brewing, these conversions occur simultaneously. Furthermore, the alcohol content differs between *sake*, wine, and beer. Wine generally contains 9–16% ABV, while most beer contains 3–9%, and undiluted sake contains 18–20% (although this is often lowered to about 15% by diluting with water prior to bottling) (Furukawa, 2011).

In Japan, the use of rice for brewing *sake* ranks second only in importance to its primary use as food. It is not produced by a conventional beer brewing process, but there are several similarities between beer and *sake* (Arendt & Zannini, 2013). *Sake* is made from steamed rice by a double fermentation process involving *koji* (*A. oryzae*) and yeast (*S. cerevisiae*) (Issara & Rawdkuen, 2016). The first step in *sake* brewing is polishing of rice. This process involves removal of the protein, lipids and minerals mainly localized in the germ and in the outer part of the rice kernel. Then the milled rice is washed, steeped in water and steamed for 30–60 min (Arendt & Zannini, 2013). The first step of fermentation is the production of a seed mash. The steamed rice is mixed with water, yeast and *koji* which is then fermented for 15 days at approximately 20°C. Due to the presence of LAB, the mash is acidified. This naturally acidified mash is mixed with water, steamed rice and *koji* at 8°C. After a few days, the mash is warmed slowly to about 15°C. The seed mash is then cooled and stored for five to seven days before it is used for the main mash. Subsequently, steamed rice, *koji* and water are added to the seed mash at 12°C. The quantity added is about twice that of the seed mash.

Difference between beer and *sake* is that for the latter the natural enzymes contained in the rice kernel are not used to break down the starch and are, in fact, deliberately inactivated before the saccharifying steps of the *sake* brewing. The solubilization of rice starch is ensured by amylolytic action of the fungal enzymes derived from *koji*, which is dominated by *A. oryzae*. *Koji* contains α- and β-amylases capable of starch liquefaction to glucose. The acidic characteristic of *koji*, resulting from either added or natural lactic acid fermentation, inhibits the growth of wild microorganisms that may compromise the quality of the fermentation process (Shurtleff & Aoyagi, 2012).

As soon as the fungal population reaches its highest concentration (10^8 CFU/g) originally present in the *koji* (generally after two days of fermentation at 12°C), one-part steamed rice and one-part water is added again. A third addition is performed the following day and the vigour of the fermentation is enhanced by increasing the temperature to 18°C. After an additional 13–17 days, the fermentation almost ceases, and the alcohol content rises to 17–

20 %, which is a world record as the highest ethanol content to be obtained by fermentation without distillation (Arendt & Zannini, 2013).

A turbid filtrate is normally obtained from the fermented mash by charcoal filtration through canvas bags. It is then usually pasteurized to kill yeast and harmful microorganisms (if present), to inactivate enzymes and to adjust the maturation velocity – a process which takes approximately three to eight months. At the end of maturation, but before the bottle-pasteurization, *sake* is blended with water to reach the appropriate alcohol content (15–16 %) and then filtered through activated carbon to adjust the colour, taste and flavour (Issara and Rawdkuen, 2016). Several indigenous rice beer-like products are produced around the world such as *sujen,* which is a popular local rice beer of Assam, India (Das, 2014).

1.3 Alcoholic beverages of Nepal

In Nepal, the history of alcoholic beverages dated back to ancient times. These technologies were developed by ethics groups while celebrating various festivals, feast and marriage ceremony (Regmi, 2007). The knowledge of home brewing has been handed down from generation to generation with little knowledge of science and technology. Among different fermented foods, *jand, raksi, toongba, nigar*, and *hyan thon* are major alcoholic beverages traditionally prepared and consumed in different part of country. Traditional alcoholic fermented starter, a *murcha* is necessary for the preparation of these beverages in Nepal, Bhutan, China and India (Tamang & Sarkar, 1995).

In traditional method of cereal fermentation, all the procedure is followed as a code of practice under unclear condition. *Murcha,* a traditional starter culture for rice wine processing. Different studies revealed that it contains both beneficial and harmful microorganisms due to lack of quality control and technical know-how of the producers. Its use has resulted in inconsistent quality of products. This study was focused on the improvement and standardization of starter culture for rice wine preparation Traditional starter causes inconsistent product quality, failure to adopt hygienic quality and controlled cultural and fermentation condition consequently loss of money and goodwill. Sanitary condition is not strictly maintained. So, there will be equal chance of successes and failure in obtaining a good product. Although these technologies are primitive, they have played a major role in socioeconomic condition of different countries people (Karki & Kharel, 2010).

Although the technology of cereal based alcoholic beverage has been known since antiquity in Nepal, but its production limited to very a small scale. To date adequate efforts have been made to commercialized traditional alcoholic beverages. One of major factor is less priority given to research and development in our country. Thus, at a time when improving of certain technologies is being recognized as a powerful tool for socioeconomic empowerment of underdeveloped countries, such as research in fermentative starter such as *murcha* is essential. Like *murcha*, in China *chu*, Korea *nuruk*, Malaysia *ragi* and Japan *koji* a series of research had been done and evolved many commercial products. But Nepalese traditional beverages face a lot of problem.

The problem in the production environment, technology, process control and nutritional status. Japanese *sake*, which is traditional product once, has now been commercialized and marketed worldwide. Traditional prepared method of *sake* is famous as premium *sake*.

Similarly, in China and Korea traditionally prepared rice wine now become commercialized and marketed worldwide.
Improvement on the traditional cereal fermentation will require certain scientific inputs such as isolation, screening, characterization, molecular analysis of isolated culture, pure culture and controlled fermentation.

1.4 Rationale

The fermentation not only enriched the diet through development of flavour and taste, but also improve the substrate biologically with protein, essential amino acids, essential fatty acids, and vitamin. Several traditional fermentation technologies from Asia and Africa have been upgraded to high technology production system because of continual efforts on research and development. Their exercise can be used to upgrade Nepalese traditional food and beverages. The defined fermented starter to carry out fermentation not only ensure safety of the product, also suitable flora in product (Karki, 2013).

In many countries, rice wine has been successfully commercialized in Japan, China, Malaysia in large scale. But in Nepal, it is produced limited household scale for their own household consumption. Now in Nepal, there are more than a dozen brands wines and beers are available in the market and has been able to capture more than thirty percent of the market. In last five years, wine consumption has grown significantly. Around one lakh bottles of Nepal wines are on demand monthly in the market (bossnepal.com/wines-nepal/). They have spent a lot of money to import raw materials from other countries. If substrate is used rice which is produced in Nepal could help to reduce overflow of a huge Nepalis currency, thereby making benefit to both industry and farmers.

In Nepal, raw material for alcoholic beverages industries mainly based on foreign countries mainly for malt, yeast and hops for beer processing and sugar pulp, yeast for wine processing. For sake processing, rice is one of the most important raw material which can be grown in Nepal, similarly yeast and mold which can be isolate from local starter culture. By using local starter culture there is change of getting most potent yeast and mold that make alcoholic beverages unique. So dependent on foreign country's raw materials can be reduced.

1.5 Scope

Development of stable fermentation starters will be in attractive proposition for use in small scale food fermentation. A good defined fermentation starter may reduce fermentation time, minimize the dry matter losses, avoid the growth of undesirable microorganism that causes off flavour in the product, finally stability and success rate in product increase. In Nepal, there is huge possibility of producing alcoholic beverages from rice (a similar product of Japanese sake). It will not only reduce overflow of currency but also develop skill and technology, utilize local raw materials, boost tourism and enhance job opportunity to Nepali people. Nepal imports 12,757,005 litres of foreign liquors worth Rs 2.20 billion. Nepal in the fiscal year 2015-16 exported 171,620 litres of alcohol worth Rs 35 million (www.b360nepal.com/e-magazines/). There is a huge different between import and export of alcoholic beverages so industrially production of rice wine is one of the ways to reduce import of alcoholic beverages.

Scientists from the Korean Food Research Institute found out that dealcoholized rice wine can cause the death of gastric cancer cells in mice (AGS human gastric adenocarcinoma cells). Tumours in this study reduced in size and volume when animals were injected with a dosage of 500 mg/kg of non-alcoholic makgeolli mixture for 7 weeks. This proves that plant extracts contained in the drink could have an anti-cancer effect (Shin et al., 2015).

1.6 Objectives

The general objective of this study was to prepare rice wine (Japanese sake-type alcoholic beverage) using domestic rice variety and mold and yeast from local sources. The specific objectives were

1.isolation and characterization of mold and yeast from *murcha* collected from different districts of Nepal,
2.selection of suitable molds (*koji)* and yeasts for fermentation of rice,
3.molecular characterization of yeast strains, and
4. potential yeast and mold isolates used for rice wine production.

1.7 Research hypothesis

By using local rice as a raw material, and molds and yeasts as a starter culture, Japanese type *sake* can be produced.

CHAPTER 2
LITERATURE REVIEW

2.1 Cereal fermented alcoholic beverages

An alcoholic drink (or alcoholic beverage) is a drink that contains ethanol, a type of alcohol produced by fermentation of grains, fruits, or other sources of sugar. Drinking alcohol plays an important social role in many cultures. Most countries have laws regulating the production, sale, and consumption of alcoholic beverages. Alcoholic drinks it is the third most popular drink after water and tea (http://www.iard.org/policy-tables/minimum-legal-age-limits/). The brewing of beer is an ancient practice of somewhere between 6,000 and 8,000 years (Bamforth, 2017). Still considered by some to owe more to art than science, it is undeniably science driven, relying on fundamentals such as pH, kinetic analysis of enzymes, use of pure strains in fermentation, and statistical tools emerging from research conducted within brewing laboratories (Wegner, 2010).

Beer like alcoholic drink is produced by Spontaneous fermentation of sugar containing any cereal due to wild yeast in air when cereal had been domesticated (Bokulich, Bamforth, 2013), Beer produced before the Industrial Revolution continued to be made and sold on a domestic scale, although by the 7th century AD beer was also being produced and sold by European monasteries. During the Industrial Revolution, the production of beer moved from artisanal manufacture to industrial manufacture, and domestic manufacture ceased to be significant by the end of the 19th century (Nicholas A Bokulich et al., 2013). The development of hydrometers and thermometers changed brewing by allowing the brewer more control of the process, and greater knowledge of the results.(Bamforth & Historical, 2008) Particularly since the latter decades of the nineteenth century, brewing has advanced to become a finely controlled technology in which consistent beer excellence can be ensured (Bamforth, 2008).

Beer is brewed from cereal grains most commonly from malted barley, though wheat, maize (corn), and rice are also used. During the brewing process, fermentation of the starch sugars in the wort produces ethanol and carbonation in the resulting beer. Most modern beer is brewed with hops, which add bitterness and other flavours and act as a natural preservative and stabilizing agent. Other flavouring agents such as herbs, or fruits may be included or used instead of hops. In commercial brewing, the natural carbonation effect is often removed during processing and replaced with forced carbonation (Wegner, 2010).

Many beer styles are classified two main types, ales and lagers, though many styles of categorization. Yeasts that ferment at warmer temperatures, usually between 15.5 and 24°C, form a layer of foam on the surface of the fermenting beer, which is why they are referred to as top-fermenting yeasts, such beers are generally classified as ales. Yeasts that ferment at considerably lower temperatures, around 10°C. These yeasts collect at the bottom of the fermenting beer and are therefore referred to as bottom-fermenting yeast (Bing et. al., 2014). Today, most of beer are produced by using bottom fermenting yeast, such as lager beer (Bamforth, 2008).

2.2 History of *sake* in Japan

History of sake making as the history of Japanese liquor or of rice-based liquor, the origin goes back as far as 2500 years ago when the rice growing become prevalent in Japan. The oldest written records about Japanese *sake* are found in the third-century in Chinese history books. These state that the Japanese have a taste for *sake* and are in the habit of gathering to drink when feel the sorrow in dead. The tenth century legal book entitled *Engishiki* records detail of ancient *sake*-making methods. At that time, *sake* was produced mainly at the imperial court, either too drunk by the emperor or for the ceremonial use (Mishra, 2016)**.**

In between 12^{th} -15^{th} centuries, *sake* came to be brewed at *Shinto shrines* and Buddhist temples, and the techniques of sake brewing today were largely developed during this period (Kitagaki and Kitamoto, 2013). This was when the brewers started using the lactic acid fermentation, making *shubo* (seed mash) used to grow yeast, relying on lactic acid to inhibit microbial contamination, and then adding the *koji,* water and steamed rice. Today, brewers had used polished rice only for *koji* production, otherwise using unpolished rice to make sake. During this period, however they started producing *Morohaku sake*, or *sake* made using the polished rice both for the *koji* rice and steamed rice added to the mash. The dairies of Buddhist priests in the 15^{th} and 16^{th} centuries record the use of hi-ire (pasteurization) with *Morohaku sake* (Portelli, 2012). Along with these advances in brewing technology, innovations in woodworking technology enabled construction of large 15,00 litres vats, facilitating the mass production of *sake*. This led to the full-fledged production of *sake* by specialists in the 16^{th} century.

2.3 Introduction of *sake*

Sake is an alcoholic beverage brewed primarily from rice and water. It resembles white wine in appearance, ranging from almost transparent to slightly yellow.

Table 2.1 Comparison of *sake*, beer and white wine in term of Physico-chemical properties

Particular	***Sake***	**Beer**	**White wine**
Alcohol (%)	13-17	4-6	10-13
Glucose (g/100 mL)	0.5-4.2	0.003-0.1	0.1-3
Etract (g/100 mL)	3-6	3-4	2-8
Nitrogen (mg/L)	700-1900	250-1000	100-900
Glutamic acid (mg/L)	100-250	10-15	10-90
Titrable acidity (g/100 mL)	0.1-0.2	0.15-0.2	0.5-0.9
pH	4.2-4.7	4.1-4.4	3.0-4.1
Succinic acid (mg/L)	200-500	40-100	500-1500
Malic acid (mg/L)	100-400	50-120	250-5000
Tartaric acid (mg/L)	0	0	1500-4000

Source: Japan *sake* and *sochu* maker association

The 13%- 17% alcohol content of many *sake* varieties is slightly higher than that of wine, but *sake* also has a mild taste with little acidity, bitterness or astringency. In terms of chemical composition, *sake* extract (consisting mostly of residual sugars) contains a high percentage of glucose and significant level of nitrogenous components and amino acids, but little organic acid (Learmonth, 2011). Careful tasting of *sake* reveals a pleasant taste that cannot be characterized as sweet, acid, bitter, or astringent. This is *umami, Umami* is sometimes described as savoriness. Compared to wine and beer, *sake* is richer in amino acids and peptides that produce *umami*. The type of *sake* known as *ginjo* has wonderfully fruity aroma. The growing popularity of *suchi* and other Japanese cuisine overseas has helped to popularize *sake* in the rest of the world. The mild flavor of *sake* also goes well with French, Italian, Chinese cuisine and it is gaining a following as a new alcoholic beverage that is distinct from wine and beer. According Japan *Sake* and *Shochu* Makers Association

2.4 Modern period of *sake* brewing process

In 1904, the national institute was established made an important contribution to the development of *sake* brewing in the subsequent years. Notably, the invention in 1909 of *Yamaha moto,* an improved version of *kimoto* style, and *sokujomoto*, which utilizes lactic acid, contributed to the stabilization and streaming of *sake* production, with the result that *sokujomoto* is now the most widely used method of producing *shubo*. Quality appraisal programs were initiated with the aim of raising the level of brewing technology in 1911 (Kitagaki & Kitamoto, 2013).

Subsequent development affecting brewing technology included breakthroughs in understanding the science of fermentation, the scientific use of microorganism, arrival of power-driven rice milling machine, a shift from wooden vats to enamel tanks and bottling of *sake* for shipment. The period during the world war second and the immediate post war period saw bold changes in producing methods, such as practice of adding the alcohol to *sake*. A wave of modernization in production processes in the 1960s and introduction machinery resulted in further streamlining. More recent trends affecting the *sake* include the notion of local production for local consumption as regional areas take another look at the skills and assets they have to offer, leading to the development of new varieties of *sake* rice and unique types *sake* yeast used in fermentation (Manfredo et al., 2017).

2.4.1 Outline of *sake* brewing

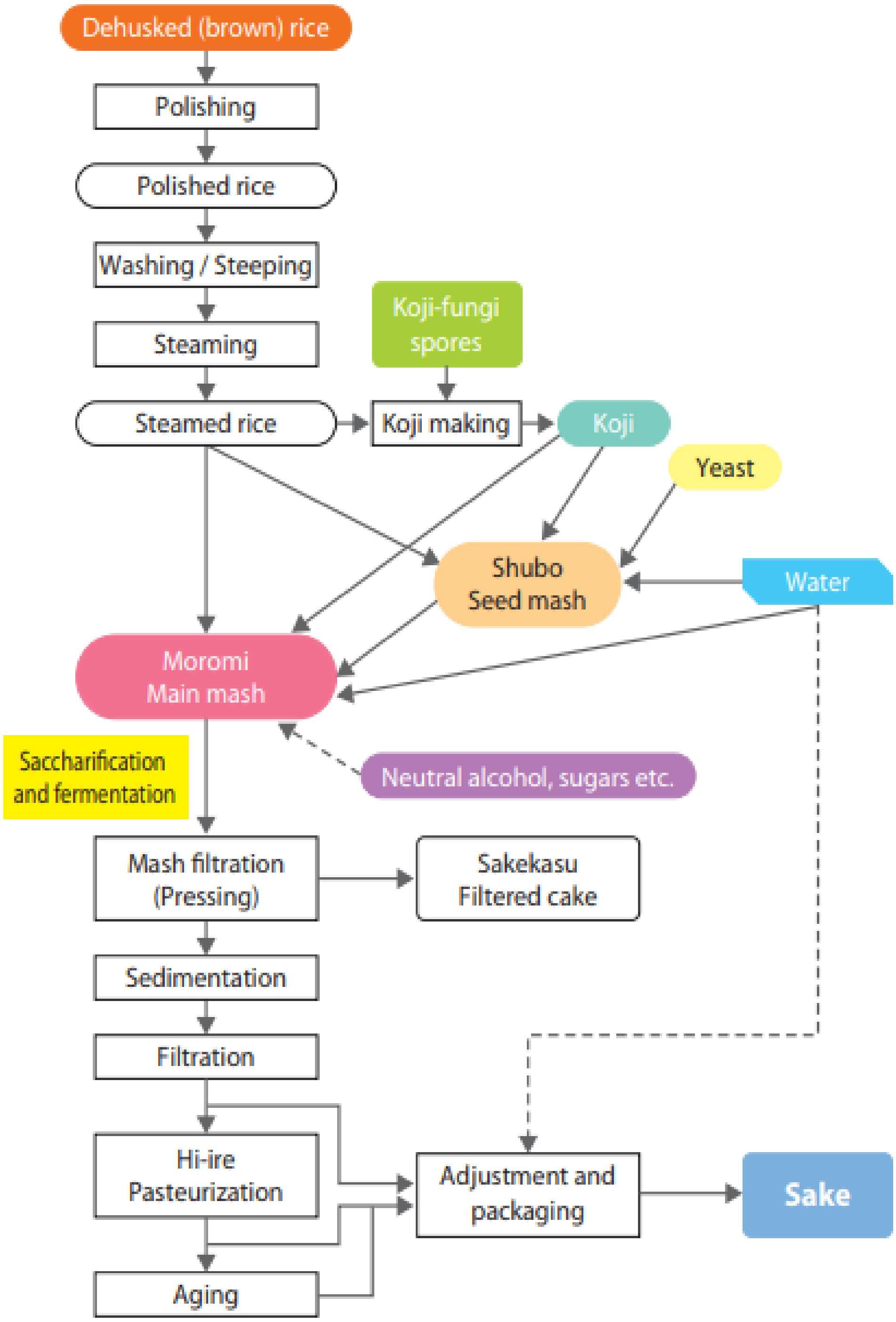
A general process of *sake* preparation according to Japan *sake* and *sochu* maker association is presented in Fig.2.1.

a) Rice and rice polishing

There are broadly two varieties of rice; Indica, a long-grained variety, and japonica, a short-grained variety. Each of these can further be subdivided into sticky and non-sticky rice. Non-sticky japonica rice grown in japan is used to brew *sake* in Japan. This is the same type of rice that Japanese people normally consume as food (Hashimoto et al., 2004). Many types of premium *sake* are made with *sake* rice, which is especially suited to *sake* brewing. Features of *sake* rice are large grains, low protein content, and high solubility during the brewing process (Arendt & Zannini, 2013).

The outer layer of unpolished rice contains large amounts of fats, minerals and proteins that spoil the favour of *sake*, therefore the rice is polished using a high-speed rotating roller.

Normally, the outer 30% of the grain is removed leaving the central 70%. This polished rice known as 70%-polished rice or is said to have a *seimai-buai* (polished ratio) of 70%. For *ginjo-shu* the outer 40% or more of the grain may be removed (Arachchige, et al..2014).



Source: Japan *sake* and *sochu* maker association

Fig. 2.1 Outline of *sake* brewing process

b) Water, steeping and steaming of rice

Japan receives abundant rainfall. Forests occupy 60% of the land surface and water is plentiful. Historically, *sake* makers directed breweries in location with access to good quality water. According to Japan *sake* and *sochu* maker association, water used to make *sake* must comply with standards applying to water for use in manufacture of food product. Importantly, it must contain no more than 0.02 ppm of iron. Too much iron gives sake reddish brown colour and spoil aroma and taste.

After milling, the polished rice is washed in water to remove the bran and is left to steep in water. When the grain has absorbed 30% of its weight in water, it is removed from the water and steamed for about one hour. Steamed rice is less moist and sticky than boiling rice, making it ideal for use in *sake* production.

c*) Koji* rice (*Kome-koji*) making

Grapes juice contains sugars, which ferment in presence of yeast, but with beverages made from grains, such as *sake* and beer, it is first necessary to use enzymes to break down the starch in the grain to convert it to sugar before yeast fermentation. The enzymes play several roles, the starch to convert it into sugar, breaking down the protein, and producing peptide and amino acids (Shurtleff and Aoyagi, 2012).

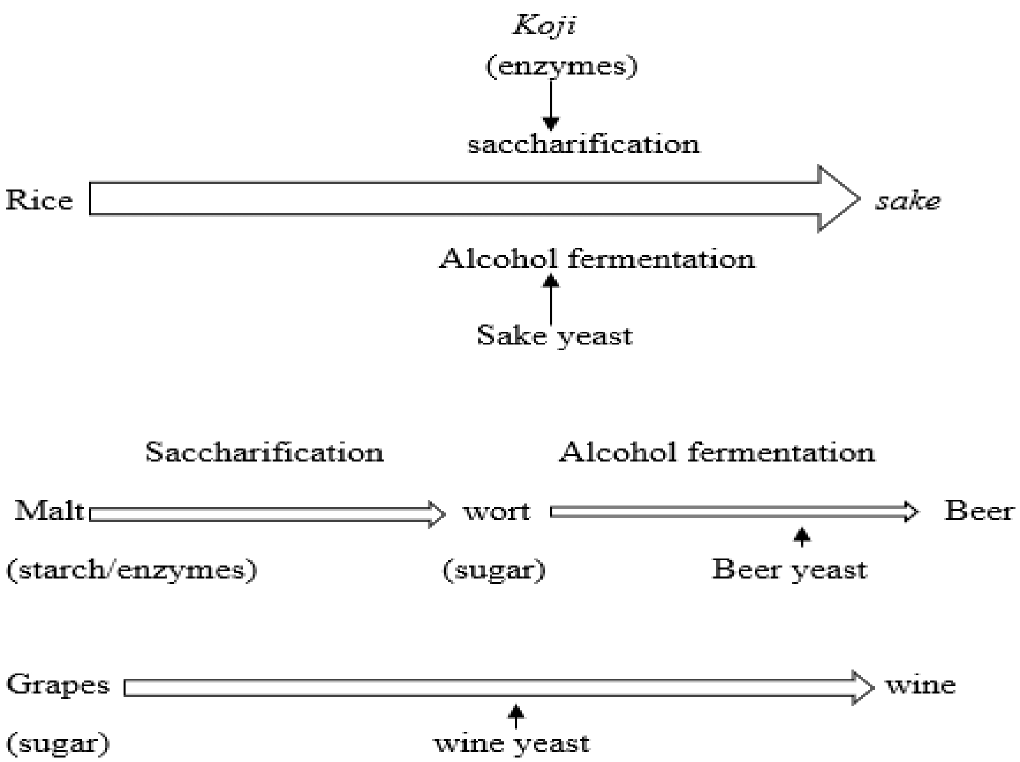


Fig. 2.2 Different in fermenting methods for *sake*, beer and wine.

In beer brewing, malt is used as the source of these enzymes, but for making *sake*, a substance called *koji* rice is used. *Koji* rice is made by cultivating *koji*-fungi (*Aspergillus oryzae)* on steamed rice. *Koji* rice may simply call *koji*. The *koji*-fungus is a beneficial and safe variety of *mold* that is also used in the production of traditional Japanese seasoning, such as *miso* and *soya sauce* (Murooka & Yamshita, 2008). The first steps in making the *koji* for use in *sake* brewing is to inoculate steamed rice with the spores of *koji*-fungi. After a while, the spores germinate and start to spread their fungal filaments. In about two days, the steamed rice is entirely covered with *koji*-fungi. As the *koji* fungal grow, they produce enzymes, which accumulate within the *koji*. *Koji*-fungi are most active at a temperature at around 36˚C but cease all activity at a temperature above 45˚C. For this reason, the process is carefully controlled in room in the brewery called a *koji-muro*, where the temperature is kept at around 30˚C and the relative humidity maintained in the range of 50%-80% (Furukawa, 2011).

d) Yeast and seed mash

Top grade yeast specifically intended for *sake* brewing is selected for the fermentation process. Before the main fermentation, the brewer first prepares seed mash, called *shubo or moto,* by significantly increasing the amount of top-grade yeast (G. Walker & Stewart, 2016). This is used as starter for fermentation of the main mash. The word *shubo* means mother of sake while the world moto means base or source. It is important for *shubo* to be highly acidic in addition to containing top-grade yeast. Fermenting in acidic condition supresses the microbes that spoil *sake*, but unlike grapes, rice itself contain no acid (Arikawa et al., 1999). That is why strongly acidic *shubo* must be used. Methods of producing highly acidic *shubo* include use of Lactic acid bacilli (LAB) and use of brewing grade lactic acid (Bokulich & Bamforth, 2013).

e) Main mash and fermentation

According to Japan *sake* and *sochu* maker association, standard ratios of steamed rice, *koji* and water placed in the fermentation tank are steamed rice 80, *koji* 20 (expressed as ratios of polished rice) and water 130. It is not all added at once, but in three steps over four days. On the first day, the amount of steamed rice and *koji* placed in the tank is equal to one-sixth of total. Seed mash (*shubo*) is also added on this first day. Nothing is added in second day, giving the yeast time to multiply. On the third day, an amount equal to two-sixths of the total is placed in tank, with the remaining three-sixths added on the fourth day. The temperature of the mix in the first step is 12˚C, but this is gradually lowered to 10˚C at the second step and 8˚C at the third step. If the entire amount were added to the tank at once, the yeast would become too diluted, prolonging the time required to reach density for the proper fermentation of alcohol and allowing microbes to multiply, which could abort the fermentation process and spoil the mixture. That is why the process is carried out in the steps.

In the sake *moromi* (main mash), the enzymes in *koji* dissolve the steamed rice and the yeast ferments the resulting sugars simultaneously in a single tank. The fermentation temperature is usually in the range of 8-18˚C. the fermentation process takes around three to four weeks, yielding an alcohol content of around 17%-20%. Using lower fermentation temperature of 12˚C or less prolongs the fermentation time to around four to five weeks. Under these conditions, the action of yeast and the process of dissolving the rice are retarded, reducing the acidity and resulting in sake with highly fruity aroma and clean taste (Walker & Stewart, 2016).

f) Mash filtration (pressing)

According to Japan *sake* and *sochu* making association, when the fermentation is complete, the *moromi* is filtered with cloth and the undissolved rice and yeast removed, leaving the new *sake*. The process may be one by placing the *moromi* in cloth bag and using machine to apply pressure from above or by using a horizontal machine like a beer mash filter press. The cake left over from the process is called *sakekasu* (filtered *sake* cake). In addition to undissolved rice and yeast, it contains about 8% alcohol by weight. S*akekasu* is highly nutritious and can be eaten as is or used as a raw ingredient for making *shochu*-traditional Japanese distilled liquor.

g) Sedimentation and filtration

With the initial filtration, some turbidity remains. If the liquid is left to stand at a low temperature, this precipitates out as sediment and the clear part is transferred to another tank. It is then filtered to produce a clear liquid. However, *sake* that has been filtered to make it clear may lose its transparency during storage. This is due to changes in the proteins dissolved in *sake* causing them to become insoluble. The use of persimmon tannin or colloidal silica is approved for removing the proteins that cause this cloudy appearance used of active charcoal is also approved for decolouring, flavour adjustment and control of the aging process (Boulton, et al., 2001).

h) Pasteurization

After sedimentation and filtration, most *sake* undergoes pasteurization (*hi-ire*) at a temperature of 60-65˚C before storing. The purpose of pasteurization is to sterilize the liquid and at the same to render any enzymes inactive (Kitamoto, et al., 1991). If the action of enzymes can continue, it increases the sweetness through the action of diastatic enzymes and alters the aroma through the action of oxidising enzymes. Many *sake* products are pasteurized again during bottling.

i) Aging (maturation)

The heating of *sake* during the pasteurization process alters the aroma and leaves it with an unrefined taste. For this reason, it can age for six months to one years. Mainly sake products are brewed between autumn and winter following the harvesting of rice, allowed to age during spring and summer (Kitamoto et al., 1991).

j) Adjustment and packaging

The alcoholic content of *sake* aged in tanks is 17%-20%, the same as at the mash filtration stage. As this level is too high for consumption with meals, brewers often add water to reduce the level to around 15% before bottling. They may also filter and pasteurize it again, if necessary.

2.4.2 Types of *sake* and their features

According to Japan's liquor tax act defines the ingredients and manufacturing process that must be used for *sake* production. The act states that *sake* must be made from rice, *koji* and water or from these ingredients plus neutral alcohol (ethyl alcohol of agricultural origin, called *jozo*-alcohol) or sugars and certain other ingredients. It also provides special designations (called *tokutei-meisho)* for *sake* that has a superior flavour and appearance and is produced in accordance with certain criteria pertaining to the ingredients and polishing. Special designations include *ginjo, daiginjo, junmai ginjo, junmai daiginjo, junmai* and *honjozo*. These currently account for around 30% of total *sake* production and can be considered premium sake. The rice used to make specially designated sake must undergo priority to ensure that it complies with required standards. For each designation, there are also standards regarding the polishing ratio and amount of neutral alcohol used. furthermore, the amount of *koji-mai* used in the production of *koji* rice must be equal to at least 15% of the total weight of polished rice used. According to Japan *sake* and *sochu* maker association, *sake* is classified as follow:

a*) Ginjo*

Ginjo-shu is made with rice grains from which more than 40% of the outer layer has been removed by milling. Fermentation occurs at lower temperature and takes longer. *Jozo-alcohol* equivalent to up to 10% of weight of the polished rice may be added. It has fruity fragrance, called *ginjo-ka,* with a light, non-acidic taste. Light does not simply mean mild or diluted. The *sake* should also have a smooth texture (mouthfeel) and good aftertaste. The specific characteristics of *ginjo-shu* vary by brewers, with the more fragrant varieties designed to highlight *ginjo-ka* and others designed with more emphasis on flavour and less on *ginjo-ka*.

b) *Daiginjo*

Daiginjo-shu is form of *ginjo-shu* made with even more highly polished rice from which at least 50% of outer layer of the grain has been removed. It has an even more refined taste and stronger *ginjo-ka* than *ginjo-shu*.

c) *Junmai, tokubetsu junmai*

Junmai-shu and *tokubetsu junmai-shu* are made only from rice, *koji* and water, highlighting the flavour of rice and *koji* more than other varieties. There are no requirements regarding polishing ratio. *Junmai-shu* is typically high in acidity and umami, with relatively little sweetness.

d) *Junmai ginjo*

Because *ginjo* brewing techniques are used in making *junmai ginjo-shu,* the acidity and umami are toned down and there is a clear *ginjo-ka*.

e) *Junmai daiginjo*

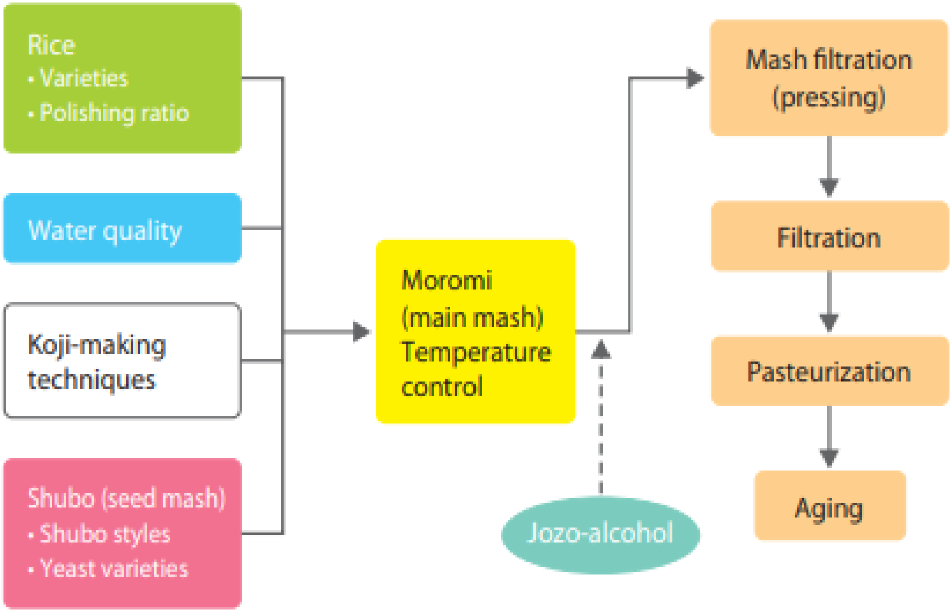
Junmai daiginjo-shu is regarded as the highest-grade *sake*. The best products in this class deliver a good blend of refined taste with acidity and umami.

f) *Honjozo*

In *honjozo-shu,* the emphasis is on flavour and there is little *ginjo-ka* or aging-induced aroma. It has a reasonable level of acidity and umami and rather than asserting the aroma and taste of *sake* itself, it helps to bring out the taste of food.

2.4.3 Factors influencing types and varieties of *sake*

A general process of *sake* preparation according to Japan *sake* and *sochu* maker association is presented in Fig 2.3.

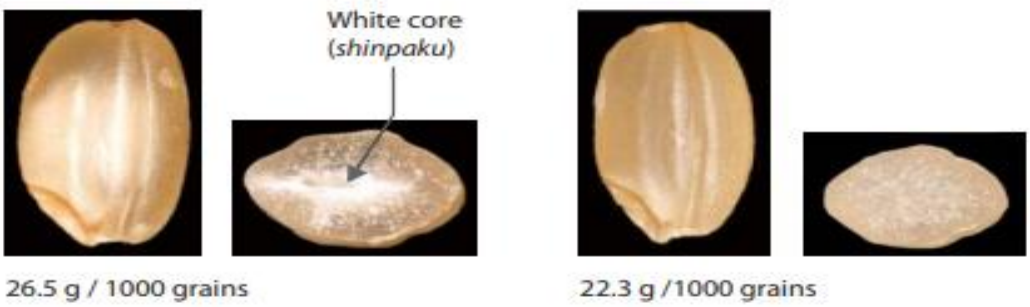


Source: Japan *sake* and *sochu* maker association

Fig. 2.3 Factors influencing types and varieties of *sake.*

a) Rice varieties

Roughly 270 varieties of japonica rice grown in Japan. These include certain varieties, known as *sake* rice, which are suitable for use in sake brewing. Sake rice grains are large and white core as well as low protein content (Kato, n.d.). The term large grain denotes any rice weighing 26 g or more per 1000 grain of rice. To be suitable for use in sake brewing, rice should be water absorbent, resilient when steamed and owing to easily penetrate by *koji*. It should also be readily soluble in *moromi* (main mash), and contain little protein, which can result in *zatsumi* (unrefined taste) if too plentiful. Sake rice has all these characteristics. Solubility level and other features of *sake* rice differ by variety, and these differences are reflected in the flavour characteristics of *sake* (Arendt & Zannini, 2013).



a) B)

Fig. 2.4 a) *Sake* rice and b) table rice.

b) Polishing ratio (*seimai-buai*) of rice

The main component of rice grain is starch, but apart from this, the outer layers and germ of unpolished rice contain many nutrients, such as protein, fats, minerals and vitamins. These nutrients are important for the proliferation of *koji*-fungi and yeast, but an overabundance speeds up the fermentation process causing the imbalance fermentation, which is detrimental to the colour, aroma, and taste of sake. For this reason, not only germ removed, but also the outer layer of unpolished rice in order reduce the level of protein, fat, minerals and vitamins. This is referred to as polishing or milling, but the amount of material removed is much greater than with polished rice for table use as shown in fig 2.5. The term polishing ratio provides an indication of how much the grain has been polished (Arachchige et al., n.d.).

c) Water

Most water in Japan is soft water, the total hardness expressed in calcium carbonate equivalent is less than 60mg/litre. Calcium stimulates the production and extraction of enzymes. Other minerals in hard water, such as potassium, magnesium and phosphates, assists the fermentation process by promoting proliferation of *koji*-fungi and yeast. For this reason, sake produced in areas where the water is hard tends to have plenty of body and dry taste with a good finish (Murooka & Yamshita, 2008).

d) Impact of rice cultivation on *sake* quality

The weather can affect the amount of rice harvested from fields. In years when the temperature is low and there is in enough sunlight at the time of panicle and grain formation, the rice grain that are smaller in size and more soluble, resulting in heavier-tasting sake than normal. In years when the weather is too hot, by contrast the starch a less soluble structure. This reduces the amount of rice that dissolves during brewing, resulting in weaker-tasting *sake*.

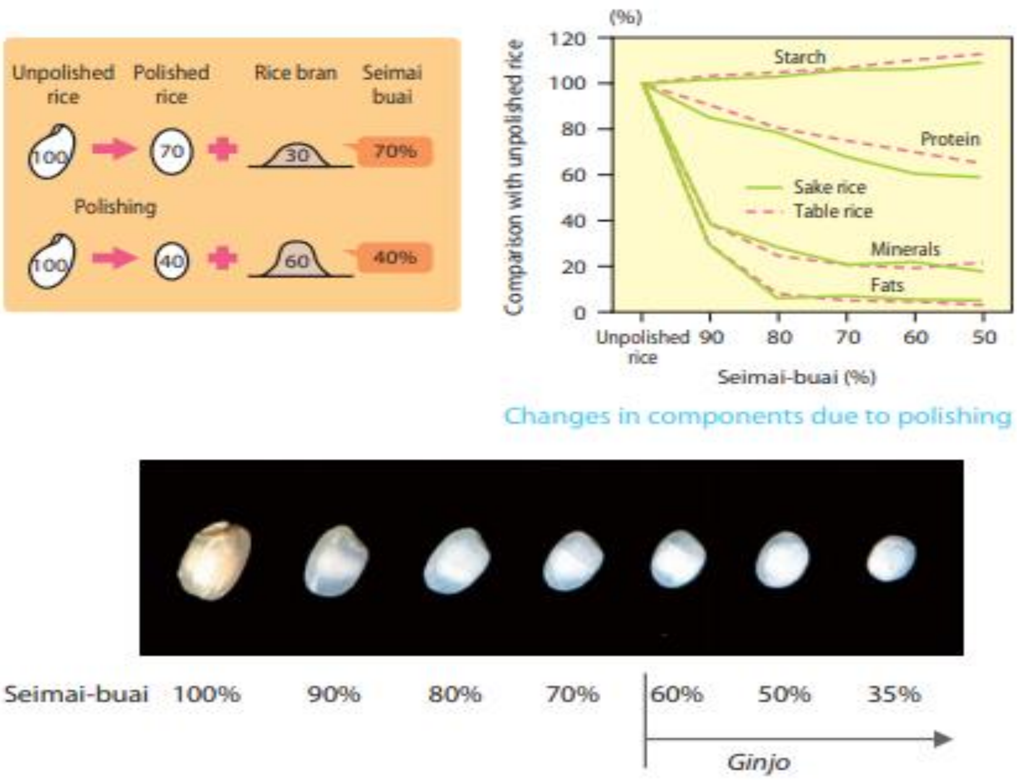


Fig. 2.5 Polishing ratio and changes in components.

e) Koji making

Unlike beer malt, *koji* is not produced in factories exclusively designed for that purpose. Each brewery makes its own *koji*. *Koji* making is the process that most exercise the mind of the *toji* (brewmaster), who oversees production of the brewery. According to Japan *sake* and *sochu* maker association, *koji* styles can be divided into *sohaze* and *tsukihaze*. In *sohaze,* the koji-fungi covers the entire rice grain sending many hyphae or strands growing into kernel. In this style, the *koji* has strong enzyme activity and the *koji* is rich in vitamins produced by *koji*-fungi. Koji made according to the *sohaze* style dissolves the rice well and promotes strong fermentation, resulting in sake with plenty of body. It is used to produce full-bodied sake and *futsu-shu* (regular *sake*) to which alcohol is added.

In the *tsukihaze* style, the *koji*-fungi grows in a spotted pattern over the rice grain. A cross section of the grain will show places where well-developed hyphae have grown into the grain and others where there are no hyphae. This still ensures appropriate enzymatic activity, but the vitamin and fatty acid content is lower. *Sake* made with this type of *koji* has a lighter taste than *sohaze sake*. *Ginjo-shu* must be produced using the *tsukihaze* style. The *toji* carefully controls the number of *koji*-fungi spores used, the quality of water and the temperature to produce *koji* exhibiting this different chrematistics.

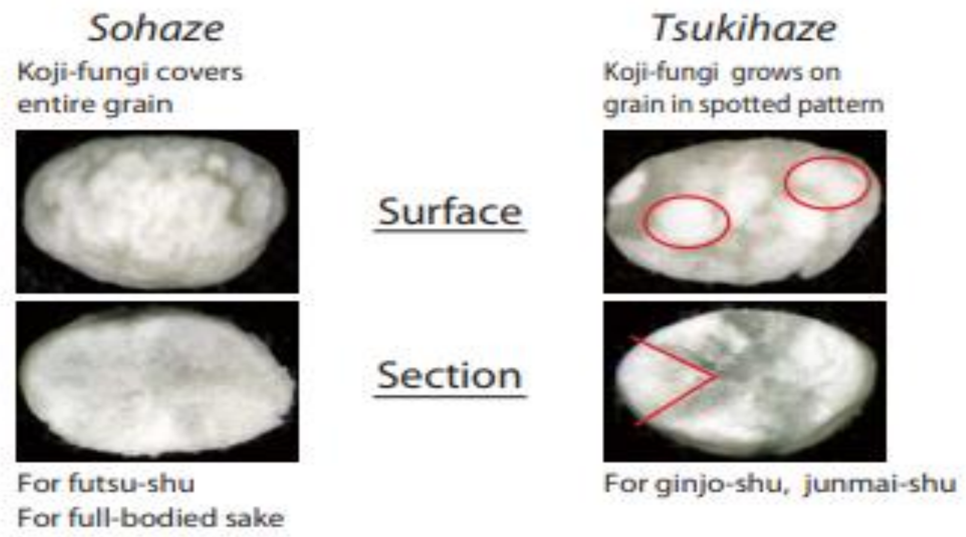


Fig. 2.6 *Koji* styles.

f) Types of yeasts

Yeast plays acritical role in determining *sake* quality. The practice of purely isolating and selecting yeast from the *moromi* of brewery that produces good sake has a long history (Arikawa et al., 1999). Since 1906, yeast selected in this manner has been distributed by of Japan (brewing society yeast). Brewing society is numbered the most widely used yeasts are #6, #7, #9 and #10. Each produces its own aroma and taste characteristics and the specific choice depends on the desired *sake* quality. More recently, brewer have been utilizing microbial technology to produce yeasts designed to increase the amount of esters delivering a fruity aroma. According to Japan *sake* and *sochu* maker association yeasts used in *sake* are:

Table 2.2 *Sake* yeast varieties

Number	Source	Characteristics
6	Aramasa shuzo (Akita), 1935	Strong fermentation, mellow flavor, suitable for creating light taste
7	Miyasaka jozo (Nagano), 1946	Vivacious flavor, suitable for ginjo and futsu-shu
9	Kumamoto-ken shuzo kenkyujo (Kumamoto), 1953	Vivacious flavor and characteristic aroma of ginjo
10	Tohoku area, 1952	Low acidity and characteristic aroma of ginjo
14	Hokuriku area, 1991	Low acidity, suitable for producing ginjo
601-1401	#6, #7, #9, #10, #14	Non-foaming yeast strains
1501	Akita, 1990	Low acidity and characteristic aroma of ginjo
1801	Breeding, 2006	Low acidity and notably fruity aroma of ginjo

Source: Japan *sake* and *sochu* maker association

g) *Shubo* (seed mash) production process

Seed mash production proce**ss**es can be divided basically into those that use lactic acid bacilli to create the lactic acid for seed mash, and processes that add brewing grade lactic acid (90% solution) directly to the seed mash (Wu et al., 2009). The processes that use lactic acid bacilli are called *kimoto* and *yamahaimoto*. The best-known process that adds lactic acid directly is called *sokujomoto.* In *kimoto* and *yamahaimoto*, only steamed rice, *koji* and water are mixed at about 8˚C. The temperature is gradually raised, and the amount of lactic acid bacilli increased. About two weeks later, once enough acid has formed, the yeast is added.

As the temperature is further raised slowly to around 22˚C, the formation of alcohol and increased the acidity of the mix the kill lactic acid bacilli, and only the yeast proliferates. It takes a month to make seed mash using this a brewing scientist to develop the *sokujomoto* process, in which lactic acid itself is added seed mash, which eliminates the need to grow a lactic acid bacilli culture and reduces the seed mash (*shubo*) preparation time by about two weeks. The *sokujomoto* process is now the most widely used. *Sake* made with the *yamahaimoto* and *kimoto* processes tends to have more complex flavour than sake made with *sokujomoyo*, because these two processes involve the use of complex microbial interactions rather than the simple addition of pure lactic acid. The resulting *sake* is said to be rich in peptides.

h) Use of *jozo-alcohol* and other ingredients

Regulations allow for the use of *jozo-alcohol* made from molasses and grains, in ginjo-shu, honjozo-shu and futsu-shu. Alcohol may be added to *moromi* used in making *ginjo-shu* and *honjozo-shu*. Normally alcohol with a concentration of 30% is used. Adding alcohol extracts aroma ingredient's derived from rice and fermentation, reducing acidity and umami to give the *sake* a light taste (Manfredo et al., 2017). In addition to *jozo-alcohol*, items that may be added to *futsu-shu* are sugars, organic acids, amino acid salts, sake, and *sakekasu*. The maximum amount of these items that can be added is less than 50% of the rice used by weight. The label must state when *jozo-alcohol* or ingredients have been used.

i) Mash filtration (pressing), secondary filtration

Once fermentation is finished, the *moromi* is squeezed to separate the *sake* from the cake. The first *sake* is released is slightly cloudy, but after this, the *sake* turns clear. The slightly cloudy *sake* that first emerges is called *arabashiri* (first run). The *sake* next released, without applying pressure, is called *nakagumi* or *nakadare*, and this is the best quality sake. The *sake* released at the end of the process after applying heavy pressure has more bitter or astringent taste (Grapes & Europe, n.d.). Some brewer fill sacks with *moromi* and suspend them to allow the *sake* to drip down. This is designed to extract the sake without applying pressure. *Sake* obtained in this manner is called *fukuodori* (sack-drip sake or *shizuku sake*). Centrifugal separation is also used at some breweries. The term *muroka* means no filtration, but at the time pressing, a cloth filter is used to separate the *sake* from cake (Kitamoto et al., 1991).

j) Pasteurization

The purpose of pasteurization is to stabilize quality by halting the action of enzymes. However, some of the freshness of freshly brewed *sake* is inevitably lost due to pasteurization (Arendt & Zannini, 2013). In recent years, advances in filtering technology and greater use of refrigerated storage and transportation have led to the marketing of growing range of unpasteurized *namazake* products relying on cold storage and transportations systems. Microfiltration is often used to remove microorganisms from *namazake*.

k) Aging of *namazake*

Sake sold as *namazake* is kept at or below 5˚C. It is stored for sixth months after production and is consumed in the spring to summer months. Prolonged storage results in a strong undesired aroma due to the enzymatic oxidation. It also gives the taste to less rough or astringent quality and boosts the sweetness, umami and body (Kitamoto et al., 1991).

l) Post pasteurization aging:

Pasteurization deactivates the enzyme and kills the yeast and other microorganism, so the only changes that occurs after pasteurization are physical and chemical. Some breweries store *ginjo-shu* and similar varieties below 10˚C, but normally *sake* is stored at room temperature. *Sake* brewed in the winter is stored over the summer, so it is consumed about one year after production.

Sake kept in long-term storage undergoes colour changes due to the Millard reaction between amino acids and sugars. There is also a decline in the fruity aroma that derives from esters and the aroma takes on sweet, burnt quality. *Sake* aged for several years and decades turns an amber or dark amber colour and the aroma becomes more complex, resembling that of soy sauce, dried fruits or nuts. In some cases, it may develop a sulfury aroma like rotten cabbage or gas. While the taste losses it astringency and sharpness, it becomes more complex and bitter. Temperature and oxygen accelerate these reactions (Amore, et al., 1989).

m) Regional factors affect in *sake* quality

Factor that determine the regional characteristics in rice, water, environment, local taste preferences and sake brewing techniques. According to Japan *sake* and *sochu* maker association regional characteristics that determine sake flavour are:

1. Rice: No single variety of rice is grown everywhere throughout the japan. Different region is suited for production of different varieties of rice.

2. Water: Most water in japan is soft water, but there are few areas where the water is hard. Dry *sake* of hard water causing feeling pleasant during drinking.

3. Environment: Plentiful snow during winter with stable low temperature and clean environment, condition that are conductive to production of sake with clean delicate taste.
4. Local taste preferences: people living in different area prefer sweeter tasting sake as well as dry tasting *sake*.

5. Sake-brewing techniques: modern *sake*-brewing techniques derive from techniques developed during 19^{th} century. As these techniques spread to different area, local variation matched to the rice, water, environment and local taste preferences of each region emerged. This technique has been handed down by regional brewing organization, giving rise to regional characteristics.

2.5 Use of starter culture for fermentation in Nepal

2.5.1 *Murcha*

Murcha is a mixed starter inoculum, used in production of local alcoholic beverages in India, Tibet, Nepal, Bhutan etc. *Murcha* is a round cake, which is mildly acidic and has a pH around 5.2 containing 13% w/w moisture and 0.7% w/w ash (dry weight basis). The *Murcha* cakes contain mixed microbial population viz. *molds*, yeasts and bacteria (Tamang & Sarkar, 1995).

Yeasts are the world's premier industrial microorganisms, which have wide exploitation in the production of foods, beverages and pharmaceuticals. The alcoholic beverages are one of the major products in the world's market. Yeasts can contaminate different dairy products because they have relatively low water activity, can easily grow at room temperature and can utilize a variety of carbohydrates (Nahvi and Moeini, 2004), e.g. pentoses, hexoses, disaccharides and, rarely polysaccharides (Barnett et al., 1990).

The capacities of the organisms, thus, can be exploited to manage the biodegradable wastes of the food, dairy and beverage industries. Most of the yeasts, except *Saccharomyces* species., can grow on cellulosic materials, however; only few genera are able to degrade starch (G. Walker & Stewart, 2016). It has been found that there are various types of *murcha* like fermentation starter form south East Asia to Africa. Which have given different names and different micro flora in each country (Ghimere & Karki 1996). '*Rogi'* prepared in Indonesia '*Budod*' in Philippines, '*Loog-pang*' in Thailand, 'Black *koji*' in Japan are very similar to Nepali *murcha* and commonly used for brewing alcoholic beverages in their own primitive way (Luangkhlaypho, et al., 2014).

In most part of Western Nepal (Syanjha, Kaski, Palpa, Parbat) *murcha* is generally prepared by certain ethnic group like *Maghi, kumal, Magar, Gurung etc*. Similarly in high mountain these are prepared by *Bhotaes* and *Sherpa* whereas in central Nepal it is common among *Newar* community. The people of Nepal use two kinds of *murcha,* the small white cake called *'manapu'* popular in western Nepal and the greenish granular pieces called *'mana'* popular in Kathmandu valley. These terms are called *kamir* or *murcha* in Nepali (Gajurel & Vaiday 1984).

The home brewers of Nepal know that the use of certain *murcha* is essential in all fermentation processes used in brewing wines and other liquors though they are quite ignorant about the broad dimension of microbial biochemistry of their complex mechanism. In fact, the exact nature of fermentation is still not fully known to them (Tsuyoshi et al., 2005).

All the organisms found in *murcha* may not be beneficial during fermentation. Number of gram-positive bacterial genera, *lactobacillus* spp. (frequent in top fermentation) and *Pedicococcus* (more common in bottom fermentation). Similarly, gram negative genera *aerobacter*, *acetobacter, acetomonas, zymomonas, oberumbacterium* are encounter in fermentation which spoil the liquor. Various wild yeast (*pitchia*, *hansenula*, *torulopsis*, and *sacchromyces*) and different types of *molds* which can produce aflatoxin also may be encounter. These entire organisms may be found in our rough and complex substances (Tamang & Sarkar, 1995).

Hence instead of using *murcha* as a fermenting starter it would be more scientific way to develop the pure culture starter of fermenting yeast isolating from *murcha* as a source to maintain quality of these products. The pure culture starter can also be commercializing instead of *murcha*. These thesis aims are to develop the pure culture avoiding the negative impacts over the ancient traditional starter culture with high fermenting capacity.

a) Yeast

Yeasts are eukaryotic, single-celled microorganisms classified as members of the fungus kingdom. The first yeast originated hundreds of millions of years ago, and 1,500 species are currently identified. oenologists have also come to recognize the importance of specific S. cerevisiae starter culture strains to the type and style of product. With the importance of S. cerevisiae's role in winemaking now firmly established, there is an ever-growing demand for new and improved wine yeast strains (Walker & Stewart, 2016).

Murcha is one of potent source from where different type of yeast can be isolated, such as *Saccharomyces bayanus*, *Candida glabrata*, *Pichia anomala, Saccharomycopsis fibuligera, Saccharomycopsis capsularis* and *Pichia burtonii.* (Tsuyoshi et al., 2005). In addition to the primary role of wine yeast to catalyze the efficient and complete conversion of grape sugars to alcohol without the development of off-flavours, starter culture strains of S. cerevisiae must now possess a range of other properties (Fleet, 2008) . The importance of these additional yeast characteristics differs with the type and style of wine to be made and the technical requirements of the winery. The need is for S. cerevisiae strains that are better adapted to the different wine-producing regions of the world with their respective grape varietals, vinicultural practices and winemaking techniques (Pretorius, 2000).

b) *Mold*

Amylases are a group of enzymes that have been found in several microorganisms like bacteria and fungi. Fungal source is confined to terrestrial isolates, mostly to *Aspergillus* species. Among the microorganisms, many fungi had been found to be good sources of amylolytic (amylase) enzymes. Studies on fungal amylase especially in the developing countries have concerted mainly on *Aspergillus niger* probably because of the ubiquitous nature and non-fastidious nutritional requirements of the organisms (Saranraj & Stella, 2013).

The major roles of fungi, mostly filamentous *molds*, in fermented foods and alcoholic beverages are the production of intra- and extracellular proteolytic and lipolytic enzymes that highly influence the flavor and texture of the product, and the degradation of antinutritive factors improving bioavailability of minerals. Species of *Actinomucor*, *Amylomyce*s, *Aspergillus, Monascus, Mucor, Neurospora, Penicillium, Rhizopus,* and *Ustilago* are reported from many fermented foods, amylolytic starters and alcoholic beverages (J. Tamang et al., 2015).

The α-amylases are calcium metalloenzymes, completely unable to function in the absence of calcium. By acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin (Zaferanloo, et al,, 2014). Because it can act anywhere on the substrate, α-amylase tends to be faster-acting than β-amylase. In animals, it is a major digestive enzyme and its optimum pH is 6.7-7. Also found in plants (adequately), fungi (ascomycetes and basidiomycetes) and bacteria ,*Bacillus* (Saranraj & Stella, 2013).

Another form of amylase, β-amylase is also synthesized by bacteria, fungi and plants (Sujeeta, et al., 2017). Working from the non-reducing end, β-amylase catalyses the hydrolysis of the second α-1,4 glyosidic bond, cleaving off two glucose units (maltose) at a time. During the ripening of fruit, β-amylase breaks starch into maltose, resulting in the sweet flavor of ripe fruit both α and β amylase present in seeds. β-amylase is present in an inactive form prior to germination, whereas α-amylase and proteases appear once germination has begun. Cereal grain amylase is key to the production of malt (Issara & Rawdkuen, 2016).

Many microbes also produce amylase to degrade extracellular starches. Animal tissues do not contain β-amylase, although it may be present in microorganisms contained within the digestive tract (Saranraj & Stella, 2013). In addition to cleaving the last α (1-4) glyosidic linkages at the nonreducing end of amylose and amylopectin, yielding glucose, γ-amylase will cleave (1-6) glyosidic linkages. Unlike the other forms of amylase, -amylase is most efficient in acidic environments and has an optimum pH of 3 (Schehl, et al., 2004).

c) Bacteria

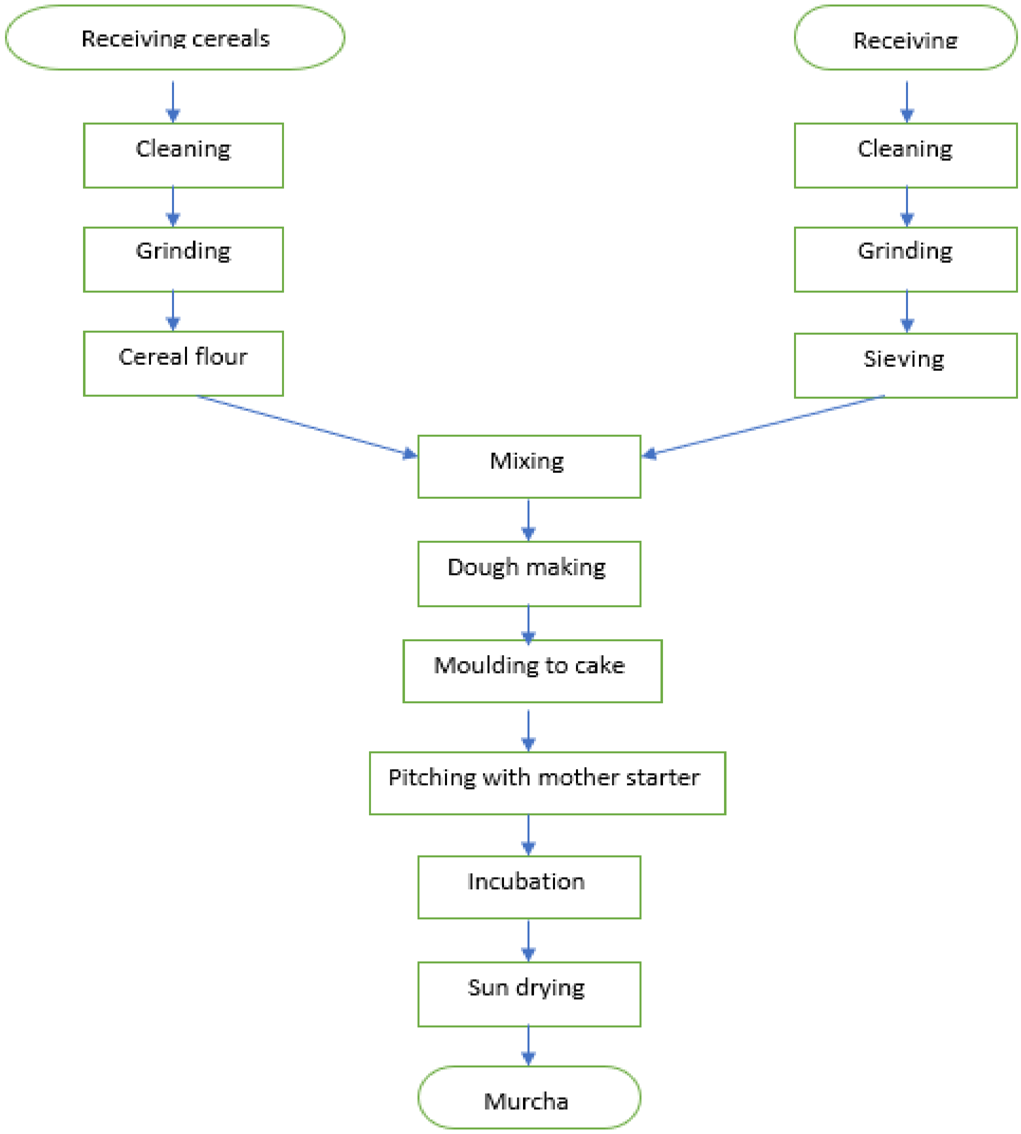
Bacteria are the most dominant microorganisms in both naturally fermented foods or foods fermented using starter cultures. Among the bacteria, lactic acid bacteria (LAB) are commonly associated with acidic fermented foods, while non-LAB bacteria such as *Bacillus, micrococcaceae, Bifidobacterium, Brachybacterium, Brevibacterium, and Propionibacterium* etc., are also involved in food fermentation, frequently as minor or secondary groups (Tamang et al., 2015)

Lactic Acid Bacteria: Lactic acid bacteria are Gram-positive, catalase-negative bacteria that produce large amounts of lactic acid. The bacterial groups that make up the LAB are among the most familiar to humans, because of their association with the human environment, and with a wide range of naturally fermented dairy products, grain crops, vegetables, and so on. The LAB comprises a large bacterial group consisting of about 380 species in 40 genera of 6 families, belonging phylogenetically to the order *Lactobacillales* within the phylum Firmicutes (Stiles and Holzapfel 1997). Common genera of the LAB isolated from various fermented foods and fermenting starter of the world are *Alkalibacterium, Carnobacterium, Enterococcus, Lactococcus, Lactobacillus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella* (Tamang et al., 2015).

2.6 Traditional starter making process for cereal fermentation in Nepal

Murcha, an amylolytic fermentation starter indigenous to Nepal, is used in preparation of alcoholic beverages such as *Jand* in Darjeeling hills and Sikkim in India, Nepal, and Bhutan. Different ethnic communities of the Nepal call it by their own language such as *Khesung* by Limbu, *bharama* by Tamang, *bopkha* or *khabed* by Rai, *phab* by Bhotia and Tibetans, *buth/thanbum* by the Lepcha. *Murcha* is produced at home exclusively by women. This art is practiced as a hereditary trade that passes from mother to daughter (Tsuyoshi et al., 2005).

Thapa (2002) has described the following method of Murcha preparation. Glutinous rice is soaked in water for 6-8 h and crushed in a food driven heavy wooden mortar and pestile. For 1 kg of rice, ingredients are added include roots of *Plumbago zeylanica* , 2.5 g; leaves of *Buddleja asiatica* Lour, 1.2 g; and previously prepared Murcha as a mother culture, 10.0 g. the mixture is kneaded, shaped into flat cakes of varying sizes, placed individually on a platform suspended below the ceiling made up of bamboo strips above the kitchen and bedded with fresh fronds of ferns (*Glaphlopteriolopsis erubescens*). The bed of the cakes is covered with dry ferns and jute bag. These cakes left to ferment for 1-3 days, sun-dried for 2-3 days and stored in a dry place for more than year.
Rai (1984) has given the following method of preparing *murcha* as follow:



Source: Rai, 1984

Fig 2.7 Traditional method of *Murcha* making in Nepal.

2.7 Some important cereal based alcoholic beverages of Nepal

a) Jand

Jand is a traditional undistilled alcoholic beverage prepared from solid-state fermentation of starchy cereals like corn, rice, wheat and millet, by using locally made starter culture known as *murcha. Jand* contains live yeasts and suspended particles and hence, classified by various workers as a category of cereal beer (Rai, 2012). *Jand* is very popular among the rural mass of Nepal (Rai, 1991). However, with the advent of alcoholic beverages based on 'new' technology, this product has earned itself social stigma. This fact notwithstanding, the annual production of *jand* is higher than that of any other indigenous fermented products and this trade is probably the single-most important economic activity among most ethnic groups of low-income categories (Subba et al., 2005).

Role of fungi in producing amylase needed to saccharify and liquefy starch. The amylase activity has been reported to reaches its peak on the second day of fermentation and mixture of yeasts (*Pichia anomala, Saccharomyces cerevisiae, Candida galbrata*) and lactic acid bacteria (*Pediococcus pentosaceus, Lactobacillus bifermentans*) in numbers exceeding 10^{5}

cfu/g in matured *jand* (Sha et al., 2017). *Jand* is a common drink for Sherpa, Bhote, Rai, Limbu, Magar, Thakali, Newar, Jyapu, Damai, Kami, etc. Now it is getting popular among Brahmin and Chhetri also (Dahal, et al., 2007). Women, especially in rural areas, prepare and sell *jand* in local market. It is an important source of income for them.(Rai, 2016).

Jand finds a prominent place in Limbu and Rai culture and other ethnic groups in general. The tradition of offering *jand* to guests is a unique way of showing hospitality. *Jand* is also used in several festive occasions, ritual rites, settling (Rai, 1991). A brief outline of *jand* preparation of *bhatte jand* (from rice) is shown in Fig. (Tamang et al., 1996). The sensory quality of *jand* is naturally dependent on its physicochemical properties, which in turn are dependent on several other factors, including the quality of *murcha*.

Glutinous rice
↓
Washing
↓
Cooking
↓
Cooling in *mandro*
↓
Inoculation with *murcha* and mixing thoroughly
↓
Placing in vessel/earthen pot and saccharification (1-2 d)
↓
Fermenting in air-tight container for 2-8 d at ambient temperature
↓
Bhatte jand

Fig 2.8 *Bhatte jand* preparation

b) *Nigar*

Nigar is the clear liquid that spontaneously accumulates during prolonged fermentation of cereal. *Nigar* can therefore, be classified as a cereal wine rather than a beer (Rai, 2006)

c) *Toongba*

Toongba is another variation of serving fermented finger millet. About 500 g of the fermented millet is transferred to a cylindrical bamboo or wooden barrel. Containing about one- fourth of hot water. The juice gradually and spontaneously is extracted and after about 15 min, the extract is sucked through a bamboo or metal pipe called *peepa*. The mash can be repeatedly steeped and sucked in to exhaust the extract (Karki, 2013).

d) *Rakshi*

Rakshi is an unaged congeneric spirit obtained by pot distillation of the slurry of fermented cereal. The product like whisky and has varying alcohol contents, generally between 15-40% (v/v). several basic researches have been done on *raksi* production from different cereals using *murcha* as well as pure culture isolated from traditional fermentation starters (*murcha*) (Dahal et al., 2007).

After the completion of fermentation, the mash is mixed with some portion of water, poured into *phosi* (a flat bottom copper, brass or aluminium pot) to about 1/3rd of its volume and *paini* (earthen pot having hole at the bottom) is placed over it. *Nani* (earthen pot for collecting distillate) is kept inside the *paini* and *bata* (condenser) is placed on top of *paini*. The *bata* is filled with cold water and firing is made. During distillation, water and other volatiles are evaporated, passed through small hoes of *paini* and condensed on the cold surface of *bata*, which in turn collected in the *nani*. The water in *bata* is changed from time to time when its temperature exceeds 45˚C as the number of water changes increases, the distillate becomes weaker in alcohol content (Dahal et al., 2007).

e) Poko

Poko is traditional rice-based beverage characterized by creamy color, soft texture, juicy and sour taste with mildly alcoholic and aromatic flavour. This product is widely used by rural people of central Nepal, especially in Kathamandu valley during occasions like wedding, festivals, cultural celebration, as well as special offering to the goddesses. Nepalese people believe that *poko* promotes good health, nourishes the body, and gives stamina.
A protocol for *poko* preparation is given in (Shrestha and Rati, 2003).

Rice
↓
Soaking for 24 h (73% moisture content)
↓
Cooking/ autoclaving and cooling
↓
Addition of starter (0.1-0.2%)
↓
Incubation at 30± 2˚ C for 5 days

Fig. 2.9 A protocol for *poko* preparation.

Although it is common dietary item, its production is confined to home scale so far. *Murcha*, the rice-based starter known as *manapu.* wheat based starters known as *mana* bring about the traditional *poko* fermentation. Mixed type of microorganisms belonging to mucorales group and yeasts followed by lactic acid bacteria are involved in *poko* fermentation (Rai, 2016).

f) *Hyaun thon*

It is undistilled alcoholic beverage indigenous to Nepal, particularly in Kathmandu valley among Newar community. It is prepared by the combination of solid and submerged state fermentation of red rice (*hakuwa*) using *mana*. The clear red colored liquid obtained after fermentation is called as hyan thon. Among the Newar community, it is one of the socially and culturally accepted alcoholic beverages and is prepared traditionally using *Hakuwa*, *mana* and water generally in the ratios of 1:1:3. A general method of preparing *hyan thon* is outlined in fig (Regmi, 2007)

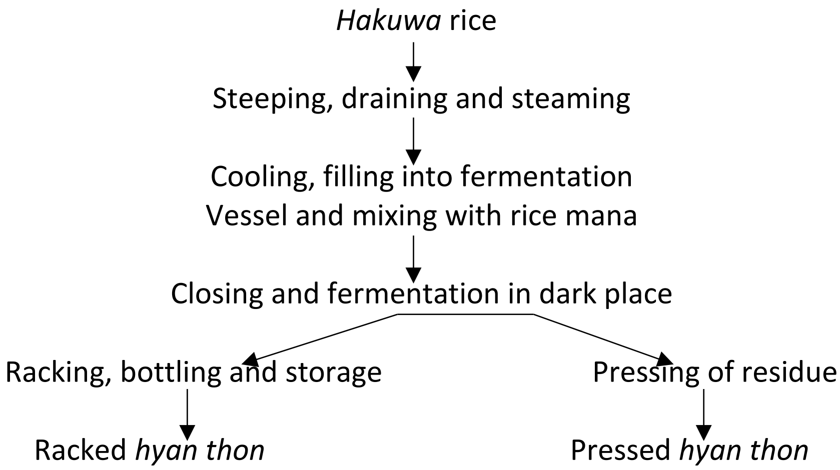


Fig. 2.10 An outline of traditional method of *hyan thon* preparation.

2.8 Physico-chemical and microbial changes during fermentation and aging

2.8.1 Esters

The production of volatile esters by yeast is of major industrial interest because the presence of these compounds determines the fruity aroma of fermented beverages, like beer and wine(Costello, et al., 2013). In alcoholic beverages, there are two important groups of esters: the acetate esters and the medium-chain fatty acid (MCFA) ethyl esters. For acetate ester synthesis, the genes involved have already been cloned and characterize(Bamforth & Lentini, 2002).

MCFA ethyl esters in beer: As they are responsible for the fruity character of fermented beverages, volatile esters are an important group of aroma compounds. The most significant flavour-active esters in beer are acetate esters of ethanol and higher alcohols: ethyl acetate (solvent-like aroma), isoamyl acetate (banana aroma), and phenyl ethyl acetate (roses, honey); and ethyl esters of MCFAs: ethyl hexanoate (apple-like aroma), ethyl octanoate (sour apple aroma), and ethyl decanoate (floral odour) (Costello et al., 2013). MCFA ethyl esters are found only in trace compounds in beer, but a certain concentration of these esters is necessary for optimum aroma and flavour. Moreover, the presence of different esters can have a synergistic effect on the individual flavours, which means that esters can also effect beer flavour well below their threshold level (Chen & Xu, 2010).

2.8.2 Aldehyde

Aldehyde are synthesized by yeast as intermediate in the fermentation of alcohol through the decarboxylation of keto acids. The majorities are further reduced by alcohol dehydrogenase, but small amount may be oxidised to acids (Tang & Li, 2016). During the active phase of fermentation, excess quantities can be excreted into the fermentation broth. The corresponding aldehyde to most of alcohols formed by yeast have been detected in alcoholic fermentation (Hazelwood, et al., 2008).

Generally, aldehyde have flavour threshold two to three orders of magnitude below the alcohols. The aroma of lower aldehyde generally perceived as fruity. Aldehyde has characteristic pungent odour. Parameter which increase the initial fermentation rate, such as aeration, readily utilize the sugars and other nutrient, higher temperature, fast fermentation yeast strain and higher pitching rates results in increased the accumulation of aldehyde (Karki & Kharel, 2011). The most abundant aldehyde was benzaldehyde, followed by furfural. The benzaldehyde, which was described as having sweet, fruity, nutty and caramel-like odours. 2-Phenyl-2-butenal, which had green, powder and cocoa aromas, was also a principal constituent of Chinese rice wine (Zuobing, et al., 2015).

2.8.3 Organic acids

Volatile acidity refers to the volatility with steam of fatty acids. The volatile acidity includes the fatty acid in series starting with acetic but exclude lactic, succinic, carbonic and sulphurous acids (Hazelwood et al., 2008). The amounts of acetic acid produced during alcoholic fermentation are small usually less than 0.03 g per 100 ml, acetic acid is normal by-product of yeast growth and has its origin primarily in the early stage of fermentation (Karki & Kharel, 2011).

To maintain the diversification and the quality of sake liquor, many efforts have been made to develop a sake related yeast in which the organic acid productivity changes. Since different sakes from malic acid, succinic acid, and lactic acid are produced during sake brewing, it is important to breed a yeast strain that shows improved productivity of organic acids and causes diversification of the taste of the resulting sake (Oba et al., 2011). Acetic acid, isovaleric acid, nonanoic acid and benzoic acid were the principal acids of the Chinese rice wine samples (Chen & Xu, 2013). Fatty acids are important for the flavour and taste of Chinese rice wine. Several fatty acids in rice wine originated from the raw materials. Most were released or produced by yeast during the fermentation process (Zuobing et al., 2015)

2.8.4 Higher alcohols (fusel oils)

Phenylethyl alcohol had a rose-honey-like flavor, and it not only served as a perfume formulation ingredient in the flavour industry but also acted as an aroma enhancer in the fermentation products (Zuobing et al., 2015). Quantitatively and qualitatively, fusel oils represent an important group of alcohols that may affect the flavour. Quantitatively, isoamyl alcohol generally account for more than 50% of all fusel oil fraction in wine (Hazelwood et al., 2008). Higher alcohols (also known as fusel alcohols) are another group of aroma compound that are greatly influenced by the different saccharifying agents. The compounds 2-methylpropanol (fusel, spirituous), 3-methylbutanol (harsh, nail polish) and 2-phenylethyl alcohol (floral, rose) are the major higher alcohols in Chinese rice wine (Chen & Xu, 2013a).

2.8.5 Microbial changes

Tamang and Thapa (2006) prepared *Bhatti jand* following traditional method using glutinous rice and local murcha. Fermentation was carried out for 8 days at 28˚C after 2 days of biomass development. They reported that population of *molds* decreased significantly ($p<0.05$) during fermentation and disappeared after the fifth day of fermentation. Population of yeasts increased significantly from 10^5 cfu/g to 10^8 cfu/g on day 2 and decreased to level of 10^5 cfu/g on day 10.

It was assumed that two types of yeast involved in the cereal fermentation: amylolytic (mostly saccharomyces) degrade starch and alcohol producing yeasts then grow rapidly on the resulting glucose to produce ethanol. Shrestha et al. (2006) studied the succession of different groups of microbes during *poko* fermentation at 30˚C using rice *manapu* and found that lactic acid bacteria increased during fermentation and were in the range of 3.5*10^6 (day 1) to 5*10^7 cfu/g (day5). A similar trend was also reported for yeast count with

1.8*10^6 and 1.3*10^8 on the 1st and 5th day of fermentation respectively. Mould counts increased from 6.3*10^5 (day1) to 1.3*10^6 cfu/g (day2).

Tamang and Thapa (2006) analysed p^H, total acidity, alcohol and reducing sugar contents during the traditional fermentation of *Bhatti jand* and found that the p^H decreased from 6.1 (day 0) to 3.96 (day 10) during fermentation at 28˚C. A large drop in p^H was recorded in the first day of fermentation (from 6.1 to3.36); there was no drastic change in p^H was reported during succeeding fermentation (0.01 to 0.11% m/m as lactic acid) and reached up to 0.17% on the 10th day of fermentation. Alcohol content increased with fermentation time. There was negligible amount of alcohol production over the 1st day of aerobic fermentation (from 0.00% to 0.2%, v/m) and reached to 10.1% v/m at the end of fermentation. Reducing sugar content increase with time attaining max of 12.6%, m/m as dextrose decrease sharply to 0.2% on day 10.

2.9 Selection criteria for yeast and *mold*

During *sake* brewing, rice starch is saccharified by enzymes produced by *koji*, *mold* (*Aspergillus oryzae*), and the resultant glucose is fermented by *sake* yeast, which produces ethanol to concentrations reaching approximately 20% (vol/vol), the highest level among nondistilled alcoholic beverages (Furukawa, 2011). *Sake* yeasts are strains of *Saccharomyces cerevisiae* and produce much more ethanol than laboratory strains of *S. cerevisiae* in sake mash.

One of the reasons for the high ethanol production of *sake* yeast is its high fermentation rate (Wu et al., 2009). This property is a critical prerequisite for sake yeast strains because rapid and high-level ethanol accumulation leads to shortening fermentation periods, as well as preventing growth of unwanted micro- organisms during sake brewing, in which open fermentation tanks are usually used. Therefore, yeast strains with higher fermentation rates have historically been selected as sake yeasts. Sake brewing is carried out at a temperature of less than 15 °C and at a low pH, enabling the yeast cells to produce more aromatic components (for example, isoamyl acetate) and inhibiting bacterial growth. As a result, yeast cells in sake mash are exposed to a very stressful environment that includes low temperature, high acidity and osmolarity, and elevated ethanol levels (Watanabe et al., 2011).

2.9.1 Ethanol tolerance in yeast

During Chinese rice wine fermentation, due to an accumulation of stressful factors, the fermentation sometimes stuck in the secondary fermentation, thus lower the quality of resultant wine (Furukawa, 2011). Though the brewing process of simultaneous saccharification and fermentation could avoid yeast cells exposing to high concentration of sugar, it also contributes to high ethanol production, which can be 14% - 20% (v/v) in final fermentation mash (Chen & Xu, 2010). Therefore, during secondary fermentation, yeast cells were face to face with so high ethanol concentration that ethanol also became toxic to yeast cells (Snoek, et al., 2016). It was a challenge for yeast to try very hard to survive and ferment.

Acquiring yeast with high ethanol tolerance was always desirable to winemakers, for which could theoretically result in more complete fermentation and higher production quality of Chinese rice wine (Schuller, 2010). It was considered that yeast strains with high ethanol tolerance increased the likelihood of exhibiting high fermentation activity, however, poor correlation between ethanol tolerance and fermentation activity had been reported (Pais et al., 2013). Although some other researchers drew conclusion that yeast with improved ethanol tolerance could get higher fermentation activity (Shi, Wang, & Wang, 2009).

Creating yeast hybrid, which the genomes of different strains were contained within one cell, to obtain yeast with superior ethanol tolerance and fermentation activity was conducted. In the present study, fermenting yeast with superior ethanol tolerance and fermentation activity was created to improve the flavour profile in Chinese rice wine. Yeast hybrids with superior ethanol tolerance and fermentation activity were screened out and then used for Chinese rice wine-making. Subsequently, the flavours profiles in Chinese rice wine fermented by yeast hybrids were investigated. Sensory evaluation was performed to assess the quality of Chinese rice wine (Yang et al., 2018).

Mechanism of ethanol tolerance is a complex phenomenon involving several hundred of genes associated with broad range of functions including protein biosynthesis, amino acid metabolism, nucleotide metabolism, transport, cell cycle and growth, lipid metabolism, fatty acid and ergosterol metabolism, membrane and cell wall organization, proline biosynthesis, and tryptophan biosynthesis (Alexandre, et al., 2001). Yeasts respond to stress induced by ethanol by accumulation of trehalose, heat-shock protein, adjusting the biosynthesis of membrane lipid composition and variation in biosynthesis phospholipids (You, et al., 2003).

Exogenous supplementation of metals ions such as calcium and magnesium ions protect the yeast cell against the ethanol stress (Birch & Walker, 2000). Also addition of zinc in the culture medium increase the ethanol tolerance (Xue, et al., 2008) by increasing the trehalose and ergosterol contents resulting in increased cell viability enhanced the ethanol production. The level of unsaturated fatty acids increase in response to increase the ethanol concentration (You et al., 2003). Amino acids such as proline exhibit multiple functions during the fermentation process including protection of cells from damage by freezing, dessication or oxidative stress by enhancing the stability of protein and membranes inhibiting the protein aggregation during protein folding (Takagi, 2008).

The limited amount of inositol results in leakage of intracellular components including nucleotide, phosphate and potassium that simultaneously affect the intracellular pH and lower the activity of H^+-ATPase, that function to maintain the homeostasis of ions in the cytoplasm, thus affecting the permeability barrier of yeast membrane (Furukawa, et al., 2004).

2.9.2 Osmotolerance in yeast

Osmotolerance is the ability to grow in an environment with a high osmotic pressure. This ability may be advantageous and economical in the biotechnological production of various compounds (Nasreen & Yaseen, et.al., 2014). Some osmotolerant yeast species are known to survive an extremely high concentration of sugars, whereas others tolerate high concentrations of salts. It is evident that the adaptation to an environment with a low water activity (aw), i.e. high osmotic pressure, may differ from species to species but is generally based on a combination of several common mechanisms, such as changes in plasma membrane composition, in redox metabolism, in the production and transport of glycerol and in the activity of various ion transporters (Thomé, 2007).

Apparently, all yeast species use glycerol as the osmolyte to compensate for the increased external osmotic pressure, but synthesized glycerol is partly lost by diffusion across the plasma membrane. One of the crucial common features of the high sugar- and salt-tolerant yeast species is their ability to efficiently transport glycerol into the cells, due to their ability to actively re transport lost glycerol (in symport with protons or sodium cations) back into the cells. This remarkable ability to accumulate glycerol produced by cells in response to osmotic pressure, but lost by simple diffusion, distinguishes osmotolerant species from osmosensitive ones and it contributes to their economical behaviour – less glycerol produced to compensate for the osmotic pressure (Hohmann, 2002). During the initial stage of fermentation, yeast cells are subjected to high osmotic conditions resulted due to high substrate concentration. As fermentation progresses, cells further encounter various conditions of changing pH, nutrient limitation and product inhibition that affects the osmotic condition of the cell resulting in cell shrinkage, reduction in cell size possibly through activation of plasma membrane mechano-sensitive ion channels and dehydration followed by decreasing cell volume and eventually loss of viability (Divate, et al., 2016).

Tolerance to osmotic shock is dependent on the physiological state of the cells. Exponential phase cell are more sensitive to osmotic stress rather than the slow growing lag phase or non-growing stationary phase cells (Mager & Varela, 1993). To avoid these adverse environmental conditions, yeast cells are known to produce polyols including glycerol, trehalose, arabitol and sorbtol(Čadež, et al., 2015).

Trehalose which acts as a membrane protectant (Divate et al., 2016) also contributes to the survival of cells when exposed to various stress conditions including osmotic stress. Trehalose, a non-reducing disaccharide, acts as a protectant against several adverse environmental conditions of heat, dessication, freeze-drying, hyperosmotic shocks, dehydration, nutrient limitation and starvation (Wiemken, 1990). High trehalose levels in yeast thus refer to increase the Osmotolerance, thermotolerance and ethanol tolerance (Shi et al., 2009).

Trehalose and glycerol accumulated in the yeast cells, the mode of accumulation however being a quite different. Glycerol is formed only during the exponentional phase of yeast growth and subsequently depleted however, trehalose levels are higher during the stationary phase and lower during the exponential phase of growth. Glycerol is said to play an important role in overcoming the osmotic stress to balance the osmotic pressure across the cell membrane and adjust the external water pressure (Divate et al., 2016).

Improving stress tolerance in yeasts (Zhao et al., 2009):

1. Single gene manipulation by overexpressing or deleting a single gene.
2. Global transcription machinery engineering (gTME) technique by simultaneous manipulation of multiple genes by introducing the randomly mutated copy of the global transcription factor that controls the transcription of large set of genes.
3. Evolutionary engineering of yeast stress tolerance to adapt yeasts to adverse environmental stresses.
4. Genome shuffling.
5. Optimization of medium composition and aeration.

2.9.3 pH Tolerance

Saccharomyces cerevisiae is the core microorganism in fermentation, especially in wine making. Most S. cerevisiae strains grow at pH values between 2.50 and 8.50, but they are acidophilic organisms and grow better under acidic condition. The optimal pH range for yeast growth can vary from pH 4.00 to 6.00, depending on temperature, the presence of oxygen, culture, and the strain of yeast (Narendranath & Power, 2005). During wine making, S. cerevisiae are always encountered with adverse environmental conditions, for example, at the beginning of fermentation, yeast cells are affected by osmotic stress because of the high sugar concentration, as well as low pH (Cardona, et al., 2007).

External environmental pH especially caused by weak acid can affect the cell wall structure and alter the conformation of proteins protruding from the plasma membrane; meanwhile, it also has an impact on the lipid organization and function of cellular membranes and the perturbation of the function of membrane embedded proteins. The loss of plasma membrane integrity increases cell permeability to ions and other small metabolites, which leads to the stimulation of passive diffusion of protons from the exterior to the cytosol (X. Liu et al., 2015) Low initial pH prolonged yeast lag phase, inhibited yeast growth, reduced fermentation rate, increased final content of acetic acid and glycerol, decreased final content of ethanol and l-succinic acid except some special cases (X. Liu et al., 2015).

2.9.4 Liquification by mold

The fungal amylases are preferred over other microbial sources because of their more acceptable GRAS (generally regarded as safe) status, the hyphal mode of growth, and good tolerance to low water activity (a_w), and high osmotic pressure conditions make fungi most efficient for bioconversion of solid substrates and thus attracting increasing attention as source of amylolytic enzymes suitable for industrial applications (Singh, et al., 2014)

Amylases are among the most important enzymes used in modern biotechnology particularly in the process involving starch hydrolysis. Fungal amylase has large applications in food and pharmaceutical industries. Considering these facts, endophytic fungi isolated from the plant *Alpinia calcarata* (Walker & Stewart, 2016). During the alcohol fermentation amyase enzyme produced by mold used to breakdown glycosidic bond in different region like reducing and nonreducing ends of starch resulting production of glucose, dextrin, maltose, maltotriose. α-amylase, β-amylase, γ-amylase used to breakdown the glycosidic bond of starch resulting production of water molecule.

CHAPTER 3
MATERIALS AND METHODS

Traditional starter culture, *murcha*, was collected from different district of Nepal (Table 3.1). those murcha sample are prepared by using local raw material apply traditional method of preparation and paddy (*Taichin*) collected from local market of Bhaktapur.

3.1 The starter culture (murcha) collection

Starter culture were collected fromdifferent districts of Nepal. These sample were coded (Table 3.1) for convenience in this research work.

Table: 3.1 Places of *murcha* collected and their code for yeast and mold culture

S. No.	Places of *murcha* collected	Code of *murcha*
1.	Lalitpur	LM
2.	Palpa	PM
3.	Sunsari	SM
4.	Dolakha	DM
5.	Parbat	ParM
6.	Ramechhap	RM
7.	Bardia	BM
8.	*Saccharomyces bayanus SN9* (commercial yeast)	CY

3.2 Identification of yeast and mold

Yeast and mold isolated from murcha sample were identified by following methods.

3.2.1 Colony morphology

All isolates aseptically cultured on the YEPDA (Appendix) agar plates PBSA (Appendix) plates for yeast and mold respectively are taken and their morphological characteristics were recorded based on colour, texture, margin, elevation.

3.2.2 Simple Staining

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

Procedure: Yeast smear was prepared on a grease free slide and heat fixed to coagulates the yeast proteins causing them to stick to the slide. Then placed on the staining tray and flooded with methylene blue for 20 – 60 seconds. The smear was gently washed to remove the excess stain. Thus, prepared slide was air dried and examine under oil immersion.

For mold cellotape method was used, for this small piece of clear tape was cut and light touch the mold colony with sticky surface. mycelial fragments and some spore were stacked to the tape. Sticky tape culture on a microscopic slight containing a drop of methylene blue and observed on microscope (Chim et al., 2015).

3.2.3 Sugar fermentation

Different sugar such as sucrose, D-glucose and starch were used to prepared plate then growth was observed after one day at 28˚C (Lee et al., 2011).

3.2.4 Urea Hydrolysis

Presence of urease and probably, its role as the major enzyme concerned in the utilization by yeasts by exogenously supplied of urea (Booth & Vishniac, 1987). Ureases catalyses the hydrolysis process to form carbonic acid and another molecule of ammonia. So, the overall reaction is a follow leading to the net increase in pH.

$NH_2 CO.NH_2 = 2H_2CO_3+2NH_3$

Procedure: Urease broth were prepared aseptically and well-marked. All the tubes were incubated at 27˚C for 24 hours and observed.

3.2.5 Starch Hydrolysis

Presence of amylase in mold used to convert starch into simple reducing sugar. The isolates were screened for starch hydrolyzing ability. The fungal isolates were inoculated on 1 % starch PBSA plate. After 3-4 days of fungal growth, the plates are flooded with iodine solution. Starch reacted with iodine to form a dark blue starch-iodine complex that covered the entire agar. When starch was broken down into sugars, there is clear zones surrounding streaked lines which indicate starch hydrolysis (Teaching and Abakaliki, 2013).

3.3 Molecular identification

Potential yeast isolates were identified by extraction of genomic DNA, PCR followed by sequencing of PCR product.

a) Extraction of gDNA from isolated yeast strain

High quality of gDNA for PCR amplification was extracted from isolated yeast strain(Garibay-Orijel, et al., 2006). Following steps were used:

1. 15 ml overnight culture of different yeast strains cells harvested by centrifugation at 12000 rpm for 1min at room temperature.

2. Pellet collected from centrifugation were mixed vigorously with 230 µl of DNA lysis buffer (2% Triton-X, 2% Sodiumdodeylsulphate, 0.1 M Nacl, 1mM EDTA, Tris-Hcl of pH 8).

3.8-10 glass beads (acidified) and 200 µl PCI were mixed in previous solution, after proper capping of eppendrof tube vortexed 30seconds.

4. Centrifugation at 12000 rpm for 5 minutes.

5. Supernatant (aquous layer) mixed with 600 µl isopropanol.

6. Incubation on ice for 30 minutes.

7. Centrifugation at 12000 rpm for 12 minutes, followed by washing 70% ethanol and dried in room temperature.

8. The dried pellet was re-suspended in 300-500 µl TE with RNAse and finally stored at -20°C for further use.

9. Gel Electrophoresis of the genomic DNA was performed in 0.8 % Agarose gel prepared in 1X TAE. Ethidium Bromide was used as the staining agent and visualization was performed on UV-trans illuminator.

b) PCR amplification of gDNA

The gDNA was amplified by using 18S rRNA primers. The sequences of forward primer and reverse primer were 5'CTTCCGTCAATTCCTTTAAG3'and 5'GGTCTTGTAATTGGAATGAG 3'(K et al., 2006). The PCR mixture was prepared in PCR tubes as (master mixture -10 µl, $MgCl_2$-0.6 µl, Forward primer (Pf)-1 µl, Reverse primer (PR-1 µl) and nuclease free water 6.4 µl). Then it was centrifuged and 1 µl template was added in each tube. After that PCR mixture were kept in PCR machine previously set at following condition.

Table 3.2 Condition for PCR amplification of gDNA

S.No.	Steps	Temperature (˚C)	Time
1.	Initial denaturation	95	2 min
2.	Denaturation	95	30 sec
3.	Annealing	51	30 sec
4.	Extension	72	70 sec
5.		Repeated steps 2-4, 30 times	
6.	Final extension	72	5 min
7.	Hold	4	Forever

After completion of PCR, gDNA was run in 1% gel-electrophoresis at 50 V for 1 hour and then it was visualized in UV. It was sent to Xcelris Labs Limited, Ahmedabad, Gujarat,India for sequencing.

c) Sequence editing and alignment

The chromatograms obtained for each region were base called using PHRED quality score (Ewing & Green, 1998b). To estimate the quality of generated sequence traces, the original forward and reverse raw sequences were assembled and edited in Sequencer v. 4.1.4 (GeneCodes Corporation, USA). Sequences were assembled based on the parameters minimum match percentage 70 and minimum overlap 20. Each Contig were viewed and manually edited (removal of gaps and dealing with ambiguous nucleotides). The aligned sequences were also edited by comparing with the reference sequence (www.ncbi.nlm.nih.gov/blast) by closely inspecting the peaks of chromatograms of forward and reverse sequence. The assembled consensus contigs were exported in text format and imported in Bioedit v.7. All candidate barcode sequences were aligned by ClusterW, (multiple sequence alignment tools) in Bioedit using default parameters. The both primer end was delineated from the alignment matrix. Primer excluded barcode sequences were exported for further analysis.

d) Phylogeny Inference

Phylogeny tree was reconstructed by Neighbor-joining (NJ) in MEGA v.7.0.14. NJ tree was constructed using P-distance as genetic measure and setting negative branch length to zero with uniform distribution rates applied. Typically, 1000 replicates of bootstrap were used to estimate tree reliability.

Node support was estimated based on the following scale: BS 50–74% (weak bootstrap support) and 75-100% for strong support (reference needed search specifically in case of bacterial)

The efficiency of tree resolution was considered successful only when the clades have at least ≥50% bootstrap value.

The sequence was deposited in the GeneBank database and accession number was obtained.

3.4 Selection criteria for yeast

Different yeast isolates were used for different tolerance test by using 1st day inoculum in fresh YEPDA media at 28˚C for one day and measured O.D at 600 nm(Lee et al., 2011).

3.4.1 Study of osmotic tolerance of yeast strains

Yeast strains were first grown in YEPDA for 24 h to obtain the inoculums. YEPD broths containing different percentage of glucose, i.e., 20, 25, 30, 35, 40, 45 and 50 were prepared in test tube from different yeast culture. Plugged by cotton plug and incubated at 28˚C for 24 h. the growth of yeasts strains in different concentrations of glucose was compared to that of the control by spectrophotometer reading at 600 nm.

3.4.2 Study of ethanol tolerance of yeast strains

Yeast strains were first grown in YEPDA for 24h to obtain the inoculums. YEPD broths containing different percentage of absolute alcohol, i.e., 10, 11, 12, 13, 14, 15, 16; were prepared in test tube from different yeast culture were sealed by cotton plug and incubated at 28˚C for 24h. the growth of yeasts strains in different concentrations of glucose was compared to that of the control by spectrophotometer reading at 600nm.

3.4.3 Study of pH tolerance of yeast strains

Yeast strains were first grown in YEPDA for 24 h to obtain the inoculums. YEPD broths containing different concentration of pH, i.e., 3, 3.5, 4, 4.5, 5 and 5.5 were prepared in test tube from different yeast culture. Plugged by cotton plug and incubated at 28˚C for 24 h. the growth of yeasts strains in different concentrations of glucose was compared to that of the control by spectrophotometer reading at 600nm.

3.5 Alcohol production test

Alcohol production test for different yeast isolates observed by Hand refractometer and dichromate oxidase method.

3.5.1 By using hand refractometer

Yeast strains were first grown in YEPDA for 24 h to obtain the inoculums. YEPD broths containing 20 percentage of glucose was prepared in test tube from different yeast culture were sealed by cotton plug and incubated at 28˚C. The alcohol produced by yeast strains was measured everyday up to 14 day of fermentation by using ERMA hand refractometer having a range of 0-30°Brix at 20°C.

3.5.2 By dichromate oxidase method

a) Ethanol estimation

1 ml of TBP and 1 ml of sample solution mixed in 2ml eppendrof tube and the vortexed vigorously using a vortex mixer for few seconds. After phase separation, 750µl of dichromate reagent was added and vortex vigorously for few seconds. After phase separation, 750 µl of dichromate reagent-containing lower phase was pipetted out for optical density measurement (Seo et al., 2009).

b) Standard graph for ethanol

Stock solution of 10 mg/ml was prepared by dissolving ethanol in distilled water. Different concentration of ethanol was prepared from stock solution, i.e., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, mg/ml. the standard graph was then plotted using the value obtained from the optical density measurement of different ethanol concentrations. The concentration of unknown sample was then obtained by plotting the values on standard curve (appendix).

3.5.3 CO_2 production test

Yeast strains were first grown in YEPD broth for 24 h to obtain from the inoculums and similarly mold isolates were first grown in PBS broth in 48 h. Then different isolates of yeast and mold were culture in conical flask containing steam rice were sealed by paraffin tape and small balloon, incubated at 28˚C for 3 day. CO_2 production were observed in balloon.

3.6 saccharification test of mold

Saccharification test of mold isolated from different murcha were observed by liquification test and Dinitrosalicyclic acid (DNS) test.

3.6.1 liquification test

Mold strains were first grown in PBSA broth for 2 day to obtain from the inoculums. then different strains of mod isolates were culture in conical flask containing steam rice were sealed by cotton plug incubated at 28˚C for 3 day. Liquification was observed in conical flask.

3.6.2 Estimation of fermentable sugars

a) Determination of reducing sugar depletion

1ml of sample was taken in test tube to which 3ml of DNS reagent was added. The sample were then incubated in a boiling water bath for 5 minutes. After incubation, the sample were incubated immediately cooled to room temperature in water and absorbance of the samples was read at 540 nm (Miller, 1959).

b) Standard graph of glucose

Stock solution of 10 mg/ml glucose was prepared and different concentration of glucose standard solution, i.e., 50, 100, 200, 400, 600, 800, 1000 and 1200 µg/ ml were made. The graph of the standard was then drawn by plotting the absorbance against the concentration at 540 nm (Appendix).

3.7 Rice wine production from selected yeast and mold isolates

Rice wine produced by using potential yeast and mold followed by selection criteria which was isolated from Parbat *murcha*.

3.7.1 Inoculum preparation

The inoculum for fermentation were prepared in YEPD broth and PBS broth of yeast and mold respectively isolated from Parbat *murcha*.

3.7.2 *koji* preparation

Koji mold is one of most important constituents during the preparation of rice wine. During mold preparation, first paddy was milled then removed bran and degree of polishing of rice was 90% similar to rice, used in rice wine preparation. Polished rice was drained in water about 4 h then autoclaving for 40min maintaining 15psi pressure at 121˚C. After autoclaving rice was placed in tray and inoculation of 5% mold prepared in PBSA media. Whole mass in room temperature for 2 days was incubated. After 2 days of incubation, sweet fruity smell was developed. Then whole mass was dried at 70˚C for 5 h. Fig. 3.1 shows the preparation of *koji.*

Koji-mold was prepared by following process:

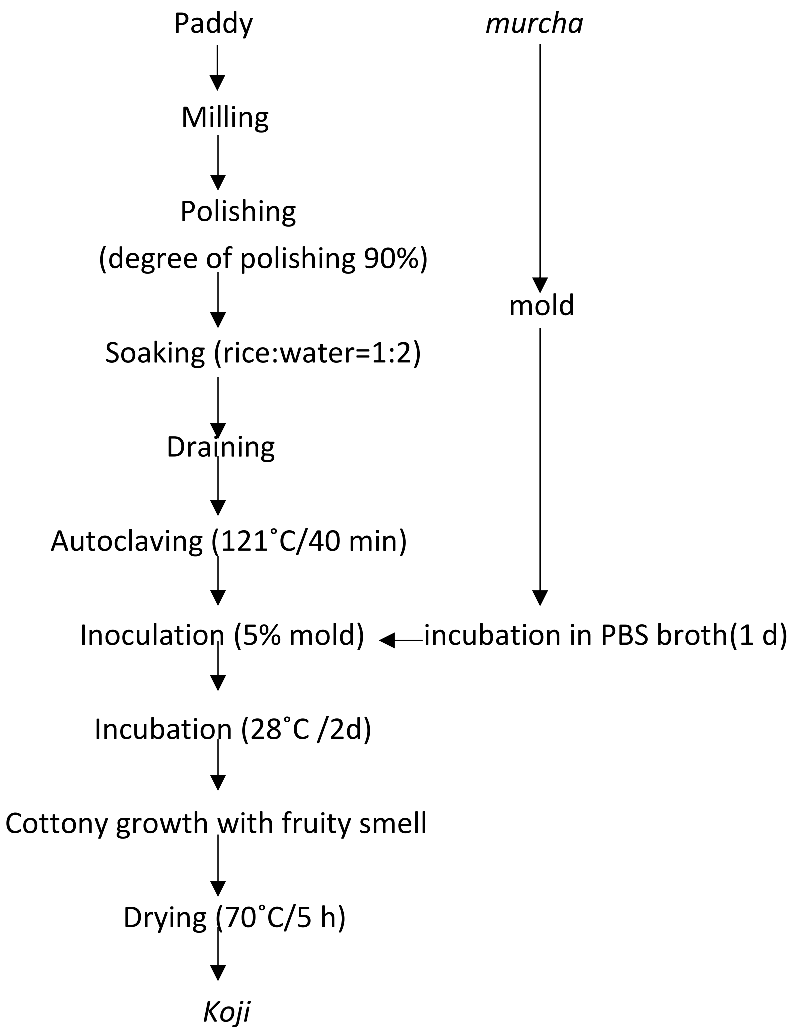


Fig 3.1 Preparation of *koji.*

3.7.3 Fermentation of rice wine

For fermentation standard ratios of steamed rice, *koji* and water placed in the fermentation tank were steamed rice 80, *koji* 20 (expressed as ratios of polished rice) and water 130. It was not all added at once, but in three steps over four days. On the first day, the amount of steamed rice and *koji* placed in the tank is equal to one-sixth of total. Seed mash (*shubo*) was also added on this first day. Nothing was added in second day, giving the yeast time to multiply. On the third day, an amount equal to two-sixths of the total was placed in tank, with the remaining three-sixths added on the fourth day. The temperature of the mix was 23˚C.

The fermentation process takes two weeks, yielding an alcohol content of around 17%-20%. Using lower fermentation temperature of 12˚C or less prolongs the fermentation time to around four to five weeks. Under these conditions, the action of yeast and the process of dissolving the rice are retarded, reducing the acidity and resulting in sake with highly fruity aroma and clean taste (Walker & Stewart, 2016). The process of fermentation of rice wine shown in Fig 3.2.

3.7.4 Clarification of rice wine

a) Racking and filtration

when the fermentation was complete, the *moromi* was filtered with cheese cloth and the undissolved rice and yeast removed, leaving the new sake. With the initial filtration, some turbidity remains. Then by, low temperature treatment this precipitates out as sediment and the clear part was transferred to another tank. Then filtered to produce a clear liquid.

b) Charcoal clarification

Finally, active charcoal was used for decolouring, flavour adjustment and control of the aging process (Boulton, et al., 2001).

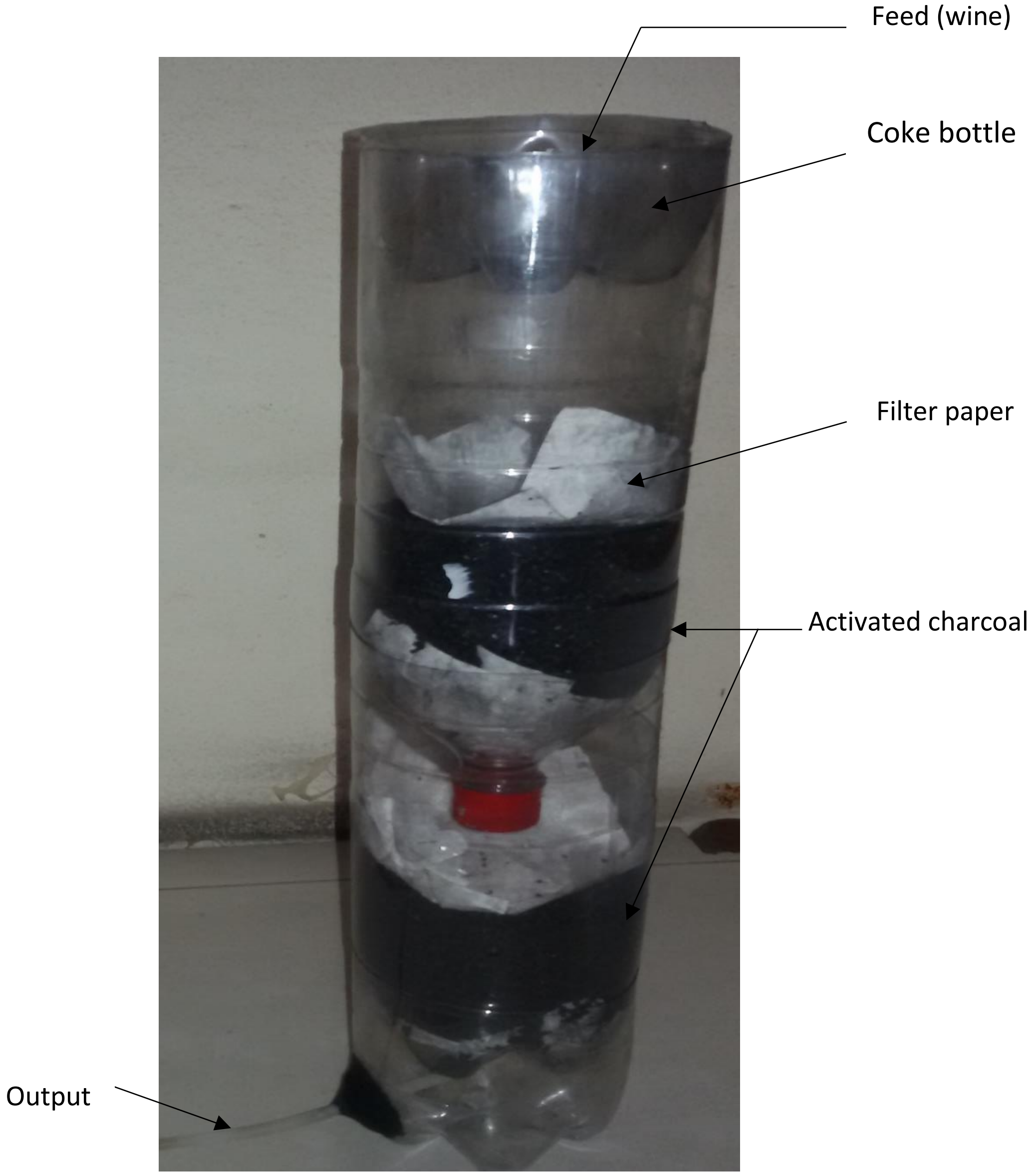


Fig. 3.2 General setup prepared for charcoal clarification.

Activated charcoal was filled in two steps. Upper first bottle was pore by using nail then covered by using filter paper and packed activated charcoal. Similarly, lower bottle was pore, cover by filter paper and packed. Bottom of second bottle was covered by another bottle as shown in Fig. Yellow turbid rice wine were passed through first and second bottle and finally collected in third bottle.

3.7.5 Chemical analysis of Rice wine

During fermentation and after treatment of charcoal of rice wine, different chemical analysis was done such as pH, titrable acidity, TSS (˚Brix), amino acid, glucose and methanol.

a) Determination of ethanol

Ethanol in sake was determine by following process:

Sake place in a beaker, flask or other container that can be used to slowly boil the sake to close to ½ its starting volume. It was important that can precisely return the level of *sake* to the pre-boil level by adding distilled water. The pre-boil amount should be close to 250mL in order to be able to properly take the specific gravity with a specific gravity bottle.

S.G. = (measured substance mass/volume)/ (distilled water mass/volume) both at 60F

After measuring both the specific gravity and the refractive index (given in Brix equivalents) with a refractometer, plug these values into the below equation then %ABV for *sake*.

Ethanol (%ABV) = $1.646 \times RI - 2.703 \times (145 - 145 / SG) - 1.794$

Where, S.G. = specific gravity; RI = Refractive Index

b) TSS (°Brix)

Brix reading of the wine samples was determined with the help of ERMA hand refractometer having a range of 0-32°Brix at 20°C.

c) pH

pH of the samples was recorded by using the pH meter of Analog model (Corion Research, USA). Standard solutions of pH 4.0 and 10.0 were used as reference to calibrate.

d) Total titrable acidity

According to *sochu* and *Sake* brewing association**;** Ten ml of the sample was taken in 100 mL volumetric flask and volume was made up. From this 10 mL of aliquot was taken in a 100 mL conical flask and titrated against 0.26M sodium hydroxide solution using one or two drops of phenolphthalein indicator. The result was expressed in term of titrable acidity as g succinic/L.

e) Amino acid

According to *sochu* and *sake* brewing association amino acid in sake was determine as follow;

First need to measure out 10 mL of sample sake to evaluate. Place this in a beaker and add a few drops of phenolphthalein. The drops are not needed if you will use a pH meter. Load the burette with about 10 mL of NaOH and record the exact amount in the burette for later reference; call it R1.

At this point, it is time to add, drop by drop the NaOH from the burette to the sample watching for the indicator to change color to a light ping for at least 30 seconds. Swirl the sample after each drop. Once the color changes and holds its light pink color for at least 30 seconds have neutralized the sample. Record the NaOH level now present in the burette; call it R2. The difference between, R1-R2 used to determine the acidity that indicate neutralize sample.

Measure 10 mL of the neutralized sample and 10mL of the neutralized formalin mixture and place in a beaker for a total of 20 mL. Reload the burette with NaOH and record the amount contained; call it R3. Titrate this 20mL sample until the color changes to a light pink for at least 30 seconds. Record the final level of NaOH in the burette; call it R4. The difference between this and the previous recording will be the amount of NaOH required to neutralize the amino acid; (R3-R4).

f) Methanol determination

Methanol of *sake* was determined by spectrophotometric process:

Procedure

i) Take 50 mL of sample in a simple still and distil, collecting about 40 mL of distillate. Dilute 1 mL of distillate to 5mL with distilled water and shaken well.

ii) Take 1 mL of this solution, 1 mL of distilled water (for blank) and 1 mL of each of the methanol standards in to 50 mL stoppered test tubes and keep them in an ice-cold water bath.

iii) Add to each test tube, 2 mL of $KMnO_4$ reagent and keep aside for 30 min.

iv) Decolourize the solution by adding a little sodium bisulphite and add 1 mL of chromotropic acid solution.

v) Mix well and add 15mL of sulphuric acid slowly with swirling and place in hot water bath maintaining 80˚C for 20 min. Observe the colour development from violet to red.

vi) Cool the mixture and measure the absorbance at 575 nm using 1cm cuvette cell.

Calculations

$$\text{Methanol (\%V/V)} = \frac{\text{Sample OD}}{\text{Standard OD}} \times 0.025 \times \text{dilution factor}$$

g) Flow chart illustrating rice wine

Flow chart of rice wine preparation as follow:

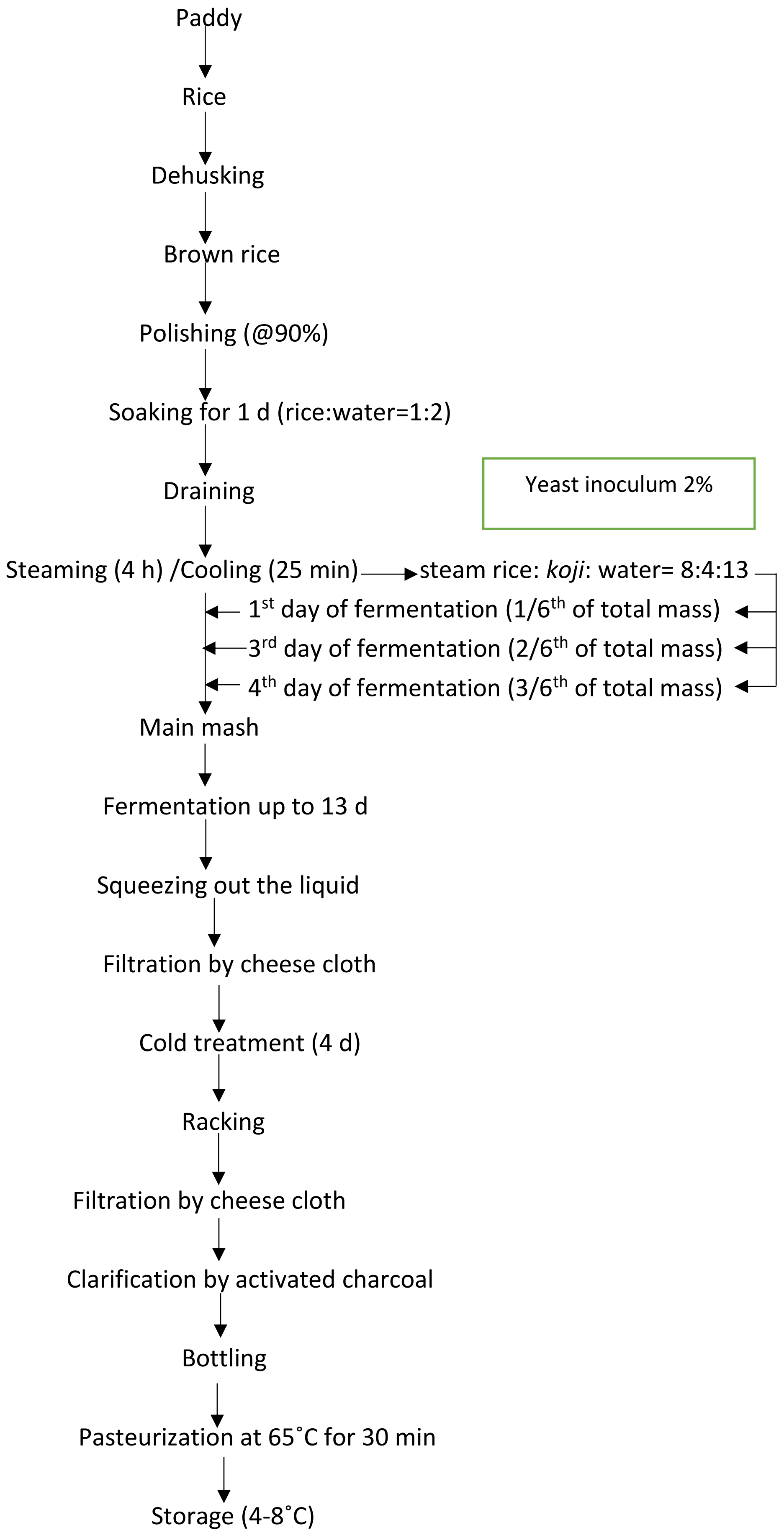


Fig. 3.3 Flow chart of rice wine preparation.

CHAPTER 4
RESULTS

Wine is a fermented beverage, generally prepared from grapes. Wine like product has also been produced by using cereals. Wine from rice is produced after saccharification of starch by microbes, enzymes (especially, commercial amylase) followed by alcoholic fermentation using yeasts. The wine quality differs with rice varieties, strains of yeast mold, processing technique, aging, etc. The experimental results on screening of fermentative yeast and mold isolates for wine preparation, nutritional and organoleptic quality of rice wines were performed. Traditional starter culture (*murcha*) samples were collected from various parts of the Nepal as presented in Table 4.1.

4.1 Enumeration of yeast in different *murcha* sample

Murcha samples collected from different part of Nepal were ground and diluted from 10^{-1} to 10^{-10}, yeasts were grown in Yeast Extract, Peptone, Dextrose and Agar (YEPDA) plate having chloramphenicol (50 ppm) at 28˚C for one day. The colonies of yeasts were counted by colony counter (Table 4.1). Among them Parbat yeast isolated from Parbat murcha had higher number of colonies per gram of murcha sample.

Table 4.1 Enumeration of yeasts in different *murcha* sample

S.N.	Sample code	Dilution factor	Colony Count (#/g)
1.	LM	10^{-8}	80
		10^{-10}	60
2.	PM	10^{-8}	85
		10^{-10}	60
3.	SM	10^{-8}	95
		10^{-10}	62
4.	DM	10^{-8}	100
		10^{-10}	70
5.	ParM	10^{-8}	105
		10^{-10}	70
6.	RM	10^{-8}	95
		10^{-10}	60
7.	BM	10^{-8}	100
		10^{-10}	56
8.	CY	10^{-8}	99
		10^{-10}	66

Note: LM= Lalitpur murcha; PM= Palpa murcha; SM= Sunsari murcha; DM= Dolakha murcha; ParM= Parbat murcha; RM= Ramechhap murcha, BM= Bardia murcha; CY= commercial yeast

4.2 Identification of yeast

Different yeast isolates isolated from different *murcha* sample were grown in YEPDA plate at 28˚C for one day and observed by naked eye (Appendix, Photo 6). The textures of the colonies were examined by touching colony using inoculating loop (Table 4.2).

Table 4.2 Morphological characteristics of yeast strain in YEPDA plate

S.N.	Sample code	Morphological characteristics
1.	LM	Mucoid, creamy colour, smooth texture
2.	PM	White colour, spongy surface
3.	SM	Mucoid, creamy white, smooth surface
4.	DM	Mucoid, creamy colour, smooth surface
5.	ParM	White colour, rough surface with white powder like substance
6.	RM	Mucoid, creamy colour, smooth surface
7.	BM	Mucoid, white colour, smooth surface
8.	CY	Mucoid, white colour, smooth surface

Note: LM= Lalitpur *murcha*; PM= Palpa *murcha*; SM= Sunsari *murcha*; DM= Dolakha *murcha*; ParM= Parbat *murcha*; RM= Ramechhap *murcha*, BM= Bardia *murcha*; CY= commercial yeast

4.3 Staining of yeast isolates

Yeast isolates isolated form different murcha were grown in YEPDA plates at 28˚C for one day were stained by methylene blue and observed at microscope (40× and 100×). All of them showed budding type of vegetative reproduction (Table 4.3.) Dead cells were stained inside whereas live cells remained unstained (Appendix, Photo 9).

Table 4.3 Morphology of yeast isolates cells and mode of reproduction

S.N.	Sample code	Shape observation	Vegetative reproduction
1.	LM	Oval	Budding
2.	PM	Oval or rounded	Budding
3.	SM	Oval	Budding
4.	DM	Mostly oval	Budding
5.	ParM	Rounded	Budding
6.	RM	Oval	Budding
7.	BM	Mostly rounded	Budding
8.	CY	Oval	Budding

Note: LM= Lalitpur *murcha*; PM= Palpa *murcha*; SM= Sunsari *murcha*; DM= Dolakha *murcha*; ParM= Parbat *murcha*; RM= Ramechhap *murcha*, BM= Bardia *murcha*; CY= commercial yeast

4.4 Carbohydrate utilization tests

Different yeast isolates isolated from different *murcha* sample were used in carbohydrate utilization test for glucose, sucrose and starch in YEPDA plates. They were incubated at 28˚C for one day and sugar utilization characteristics were observed. All of them used glucose and sucrose Ramechhap yeast isolates that used glucose (Table 4.4). As expected, none of them utilized starch for their carbon source as indicated by failure of colonies formation.

Table 4.4 Carbohydrate use of different isolates of yeast

S.N.	Sample code	D-Glucose	Sucrose	Starch
1.	LM	+	+	-
2.	PM	+	+	-
3.	SM	+	+	-
4.	DM	+	+	-
5.	ParM	+	+	-
6.	RM	+	-	-
7.	BM	+	+	-
8.	CY	+	+	-

Note: + indicates positive growth; - indicates no-growth

LM= Lalitpur *murcha*; PM= Palpa *murcha*; SM= Sunsari *murcha*; DM= Dolakha *murcha*; ParM= Parbat *murcha*; RM= Ramechhap *murcha*, BM= Bardia *murcha*; CY= commercial yeast

4.5 Urea hydrolysis test

Urea hydrolysis test showed presence or absence of urease producing capacity of yeast isolates. Presence of urease enzyme, indicates utilization of urea as nitrogen source. On urea hydrolysis test, all the isolates of yeasts showed negative results for 1 day of incubation at 28˚C (Fig. 4.1).

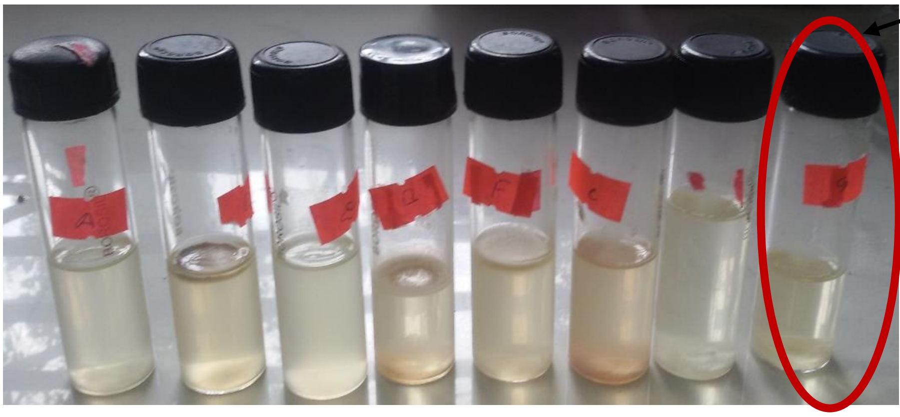


Fig.4.1 Urease test of different yeast isolates in Urease broth.

4.6 Morphological characteristics of mold

Mold isolates isolated from different *murcha* samples were grown in peptone, beef extract, starch agar (PBSA) media at 28˚C for 2 days (Appendix, Photo 7). All of them showed filamentous type of structure by naked eye except mold isolated from Lalitpur and Dolakha. Lalitpur and Dolakha yeast isolates showed mucoid with solid surface (Table 4.5). Similarly, all of them showed sporangiophore with sporangium during microscopic observation by methylene blue staining and observed in microscope in 40× (Appendix, Photo 8) using cellotape method.

Table 4.5 Morphological and microscopic characteristics of mold

S.N.	Sample code	Morphological Characteristics	Microscopic Characteristics
1.	LM	Mucoid with solid surface	Sporangiophore with sporangium
2.	PM	Filamentous	Sporangiophore with sporangium
3.	SM	Filamentous	Sporangiophore with sporangium
4.	DM	Mucoid with solid surface	Sporangiophore with sporangium
5.	ParM	Filamentous	Sporangiophore with sporangium
6.	RM	Filamentous	Sporangiophore with sporangium
7.	BM	Filamentous	Sporangiophore with sporangium

Note: LM= Lalitpur *murcha*; PM= Palpa *murcha*; SM= Sunsari *murcha*; DM= Dolakha *murcha*; ParM= Parbat *murcha*; RM= Ramechhap *murcha*, BM= Bardia *murcha*

4.7 Starch hydrolysis

Mold isolates isolated from different *murcha* samples were grown in PBSA media at 28˚C for 2 days. Iodine (10% solution) was floated on the PBSA plates containing different mold, and color was observed after 30 seconds. Halo zone near the filamentous structure of mold indicated presence of amylase. All the isolates of mold had positive result to the starch hydrolysis showing the presence of amylase enzyme (Fig.4.2).

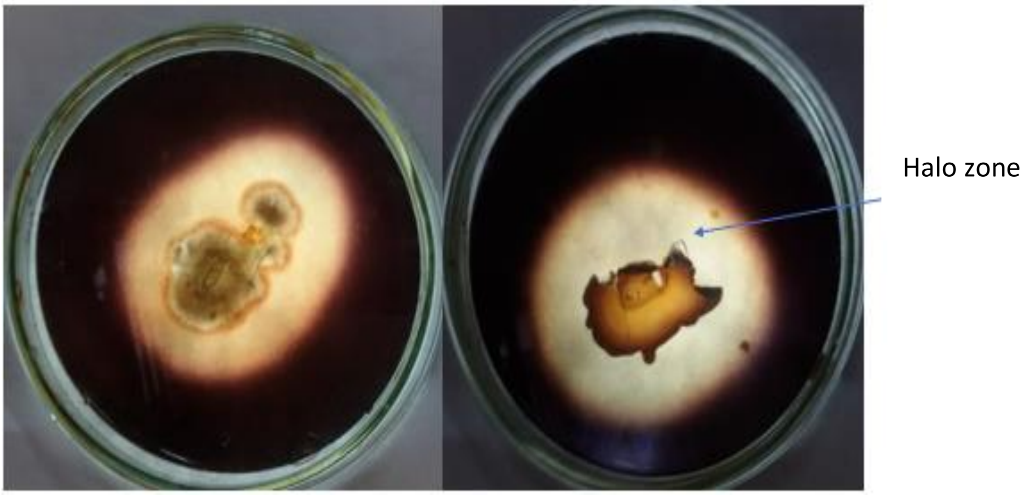


Fig.4.2 Halo zone on PBSA media.

4.8 Selection criteria of yeast

During wine fermentation process simple sugars are utilized to produce ethanol and CO2. In the fermentation process, yeast faces different stresses such as osmotic pressure causes by high concentration of sugar, ethanol, pH, sulphur dioxide that should be monitored and control for efficient fermentation. In each test first day, maintained 0.4 optical density at 540 nm. Then all of test are performed in second day.

4.8.1 Sugar tolerance test

Seven yeast strains were isolated from 7 different *murcha* samples. First of all, yeast isolates that grew on YPD agar plates with 20% dextrose were selected. Then sugar tolerance tests were performed with YEPD broth media with 20, 25, 30, 35, 40, and 45% dextrose at 28˚C for one day. Growth was examined at 600 nm. All isolates showed good result up to 40% of glucose in media. Parbat yeast showed better growth among other isolate including commercial yeast (Fig. 4.3).

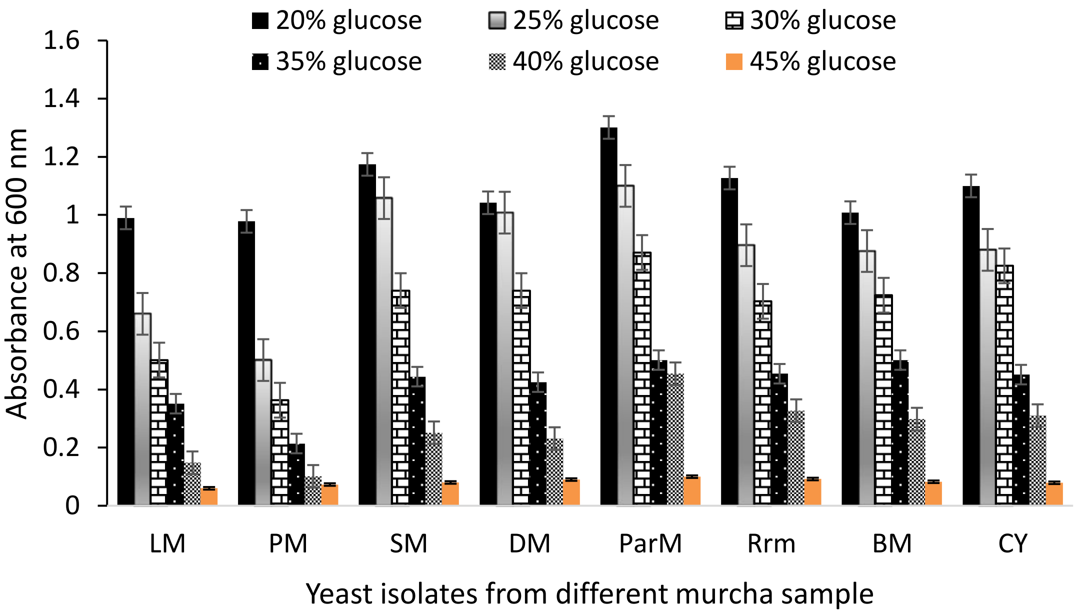


Fig. 4.3 Tolerance of yeast isolates at different concentration of glucose. Vertical bars indicate standard error.

LM= Lalitpur *murcha*; PM= Palpa *murcha*; SM= Sunsari *murcha*; DM= Dolakha *murcha*; ParM= Parbat *murcha*; RM= Ramechhap *murcha*, BM= Bardia *murcha*; CY= commercial yeast

4.8.2 Alcohol tolerance test

Seven yeast strains were isolated from 7 different *murcha* samples. First of all, yeast isolates that grew on YPD agar plates with 10% ethanol by volume were selected. Then ethanol tolerance were performed with YEPD broth media with 10 to 15% ethanol by volume at 28˚C for one day. Growth was examined at 600nm. All isolates showed alcohol tolerance up to 13%. Yeast isolate from Parbat *murcha* showed high alcohol tolerance in different percentage of alcohol (Fig. 4.4).

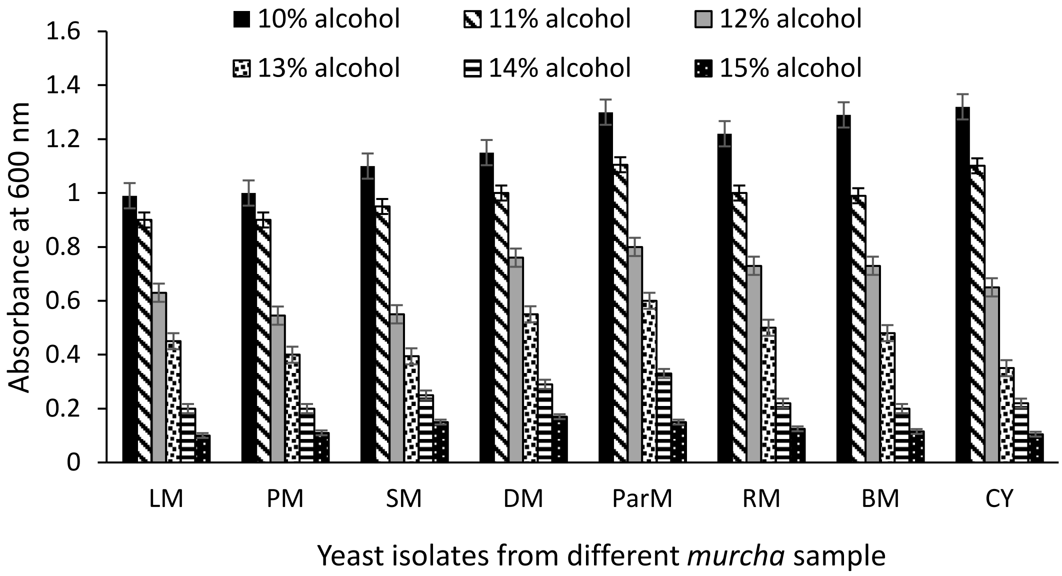


Fig. 4.4 Tolerance of yeast isolates at different alcohol concentrations. Vertical bars indicate ± standard error.

LM= Lalitpur *murcha*; PM= Palpa *murcha*; SM= Sunsari *murcha*; DM= Dolakha *murcha*; ParM= Parbat *murcha*; RM= Ramechhap *murcha*, BM= Bardia *murcha*; CY= commercial yeast

4.8.3 pH tolerance test

Seven yeast strains were isolated from 7 different *murcha* samples.

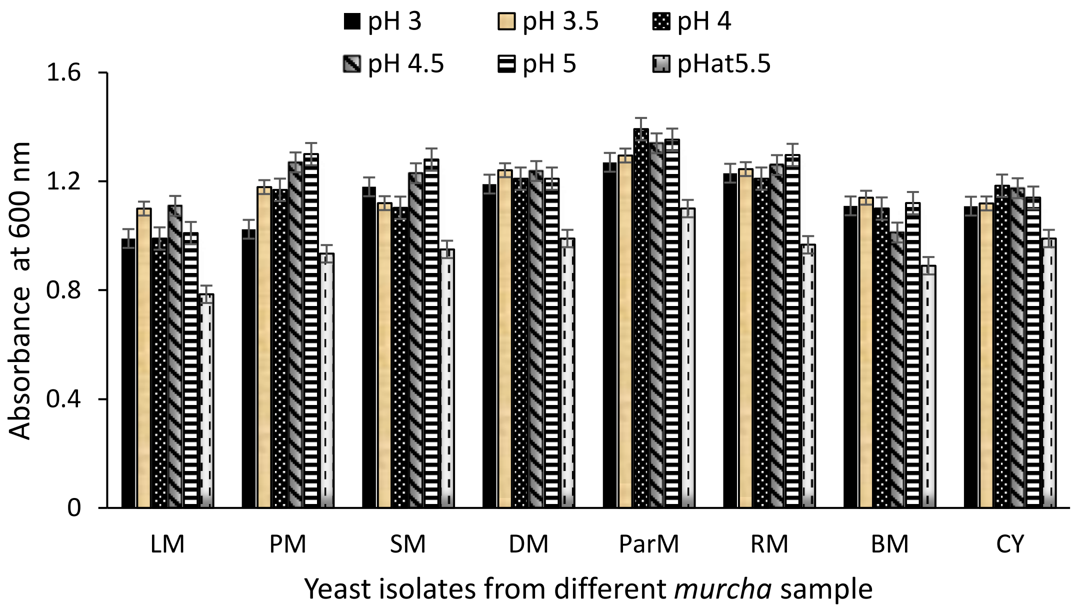


Fig. 4.5 Tolerance yeast isolate at different pH. Vertical bars indicate ± standard error.

LM= Lalitpur *murcha*; PM= Palpa *murcha*; SM= Sunsari *murcha*; DM= Dolakha *murcha*; ParM= Parbat *murcha*; RM= Ramechhap *murcha*, BM= Bardia *murcha*; CY= commercial yeast

First of all, yeast isolates that grew on YPD agar plates with 4 pH were selected. Then pH tolerance were performed with YEPD broth media with 3 to 5.5 pH at 28˚C for one day. Growth was examined at 600 nm. All isolates showed similar type of tolerance to pH, among them high pH tolerance showed by isolate of Parbat yeast (Fig. 4.5). All of them showed best growth at pH at 4 to 5.

4.8.4 PCR amplification results of yeast isolates

All isolates of yeast isolated from different murcha sample grow in YEPD broth at 28˚C for one day and maintaining OD 0.4 at 600 nm.

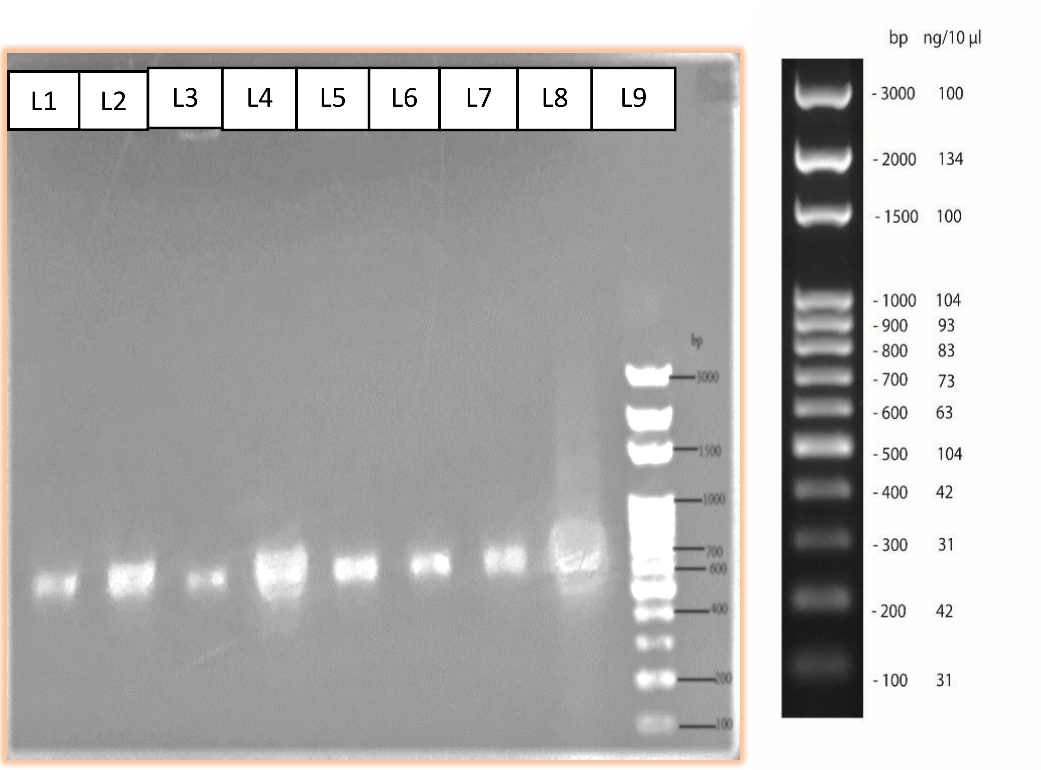


Fig. 4.6 PCR products of yeasts isolates from different *murcha* sample with ladder of 100 bp (Solis Biodyne).

Note: L1= Lalitpur yeast; L2= Palpa yeast; L3= Sunsari yeast; L4= Dolakha yeast; L5= Parbat yeast; L6= Ramechhap yeast; L7= Bardia yeast; L8 =Commercial yeast; L9= Ladder

And genomic DNA was extracted from culture of yeast. After gel run, DNA band of yeasts were observed in gel dock. Then PCR of all sample of yeast was done by using primer under standard conditions and bands were observed in gel dock (Fig. 4.6). All of yeast isolates showed similar type of DNA bands that lied in between 600 to 700 bp by comparing with ladder (100 bp) of Solis Biodyne. For further confirmation of yeast, PCR product of two yeast from Dolakha and Parbat were sent for sequencing.

4.8.5 Molecular identification of potential yeast for fermentation

Based on selection criteria of yeast isolates, Parbat yeast isolate showed most potent character among seven yeast isolates. So further analysed of yeast from Dolakha *murcha* (Fig. 4.7 and 4.8) and Parbat *murcha* (Fig. 4.9 and 4.10) was done by sequencing. The sequence of Isolatates were obtained from Xcelris Lab, India and further BLAST was done to find the most probable genus of fungus.

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	Accession
☐	Saccharomyces cerevisiae strain Y169 chromosome 12	1047	2094	96%	0.0	99%	gi|1511320837|CP033481.1
☐	Saccharomyces cerevisiae strain X55 chromosome 12	1047	2094	96%	0.0	99%	gi|1511320820|CP033498.1
☐	Saccharomyces cerevisiae strain KSD-Yc chromosome 12	1047	7230	96%	0.0	99%	gi|1497470364|CP024006.1
☐	Saccharomyces cerevisiae strain Axenic culture small subunit ribosomal RNA	1047	1047	96%	0.0	99%	gi|1468800195|MH822527.1
☐	Saccharomyces cerevisiae strain HBUM07162 18S ribosomal RNA gene, par	1047	1047	96%	0.0	99%	gi|1409189685|MF662311.1
☐	Saccharomyces cerevisiae strain HBUM07160 18S ribosomal RNA gene, par	1047	1047	96%	0.0	99%	gi|1409189683|MF662309.1
☐	Saccharomyces cerevisiae strain HBUM07159 18S ribosomal RNA gene, par	1047	1047	96%	0.0	99%	gi|1409189682|MF662308.1
☐	Saccharomyces cerevisiae strain HBUM07157 18S ribosomal RNA gene, par	1047	1047	96%	0.0	99%	gi|1409189680|MF662306.1

Fig. 4.7 Blast result showing percentage identity of Isolate of Dolakha *murcha* with other *saccharomyces cerevisiae.*

Molecular Evolutionary Genetics Analysis (MEGA) was used as a tool for conducting sequence alignment. Those aligned sequences were used to make phylogenetic trees. The analyses used to construct phylogenetic tree were conducted with the following specifications: Nucleotides were used as substitutions type, maximum likelihood was used as the statistical method, bootstrap method with 2000 bootstrap replications was used to test phylogeny. Sequence similarity searches was performed for the fungal sequences against the nonredundant database maintained by the National Center for Biotechnology Information (NCBI) using the Basic Local Alignment Search Tool (BLAST) as the tool to infer functional and evolutionary relationships between sequences as well as to help identify members of gene families. The obtained sequence showed maximum similarity with *Saccharomyces cerevisiae.*

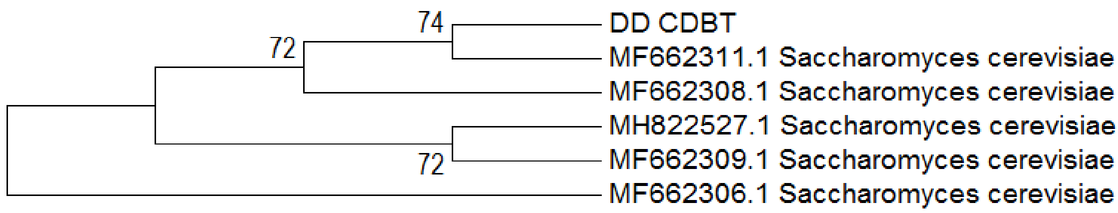


Fig. 4.8 Phylogenetic tree of yeast isolate of Dolakha *murcha.*

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

	Description	Max score	Total score	Query cover	E value	Ident	
☐	[Candida] glabrata ribosomal RNA (CAGL0L13398r), rRNA	1045	1045	97%	0.0	99%	gi|1246395
☐	Saccharomyces sp. 'boulardii' voucher URCS6 18S ribosomal RNA gene, parti	1045	1045	97%	0.0	99%	gi|1008981
☐	Saccharomyces sp. 'boulardii' voucher URCS5 18S ribosomal RNA gene, parti	1045	1045	97%	0.0	99%	gi|1008981
☐	Saccharomyces sp. 'boulardii' voucher URCS4 18S ribosomal RNA gene, parti	1045	1045	97%	0.0	99%	gi|1008981
☐	Saccharomyces sp. 'boulardii' voucher URCS3 18S ribosomal RNA gene, parti	1045	1045	97%	0.0	99%	gi|1008981
☐	Saccharomyces sp. 'boulardii' voucher URCS1 18S ribosomal RNA gene, parti	1045	1045	97%	0.0	99%	gi|1008981
☐	Saccharomyces cerevisiae strain KS10 18S ribosomal RNA gene, partial sequ	1045	1045	97%	0.0	99%	gi|9529788

Fig. 4.9 Blast result showing percentage identity of Isolate of Parbat *murcha* with other *saccharomyces cerevisiae.*

MEGA was used as a tool for conducting sequence alignment. Those aligned sequences were used to make phylogenetic tree. The analyses used to construct phylogenetic tree were conducted with the following specifications: Nucleotides were used as substitutions type, maximum likelihood was used as the statistical method, bootstrap method with 2000 bootstrap replications was used to test phylogeny. Sequence similarity searches was performed for the fungal sequences against the nonredundant database maintained by the NCBI using the BLAST as the tool to infer functional and evolutionary relationships between sequences as well as to help identify members of gene families. The obtained sequence showed maximum similarity with *Saccharomyces boulardii.*

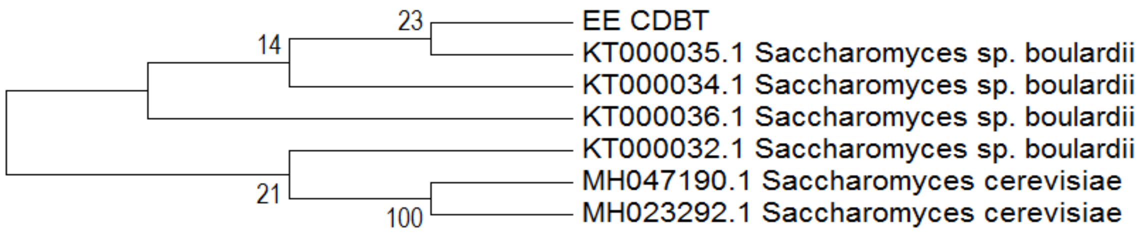


Fig 4.10 Phylogenetic tree of yeast Isolate of Parbat *murcha.*

After submission of sequence in GeneBank, got an accession number from GenBank, MK253787 and MK253281 for yeast isolates isolated from Dolakha and Parbat *murcha* respectively.

4.9 Alcohol production test

Alcohol produce by different yeast strain during incubation in YEPD broth at 28˚C for one and 14 days of fermentation by using dichromate Oxidase method and by using hand refractometer.

4.9.1 Alcohol production test using hand refractometer

Yeast isolates isolated from different *murcha* sample were grown in YEPD broth at 28˚C for 12 days and changes in total soluble solid (˚Bx) were measured (Fig. 4.11). Decrease in ˚Brix indicates tentative ethanol (% by vol.) in fermentation process. Changes in total soluble solids (˚Brix) were recorded for all test fermentations along with fermentation with commercial yeast *(S. bayanus* SN9) under same condition for 12 days.

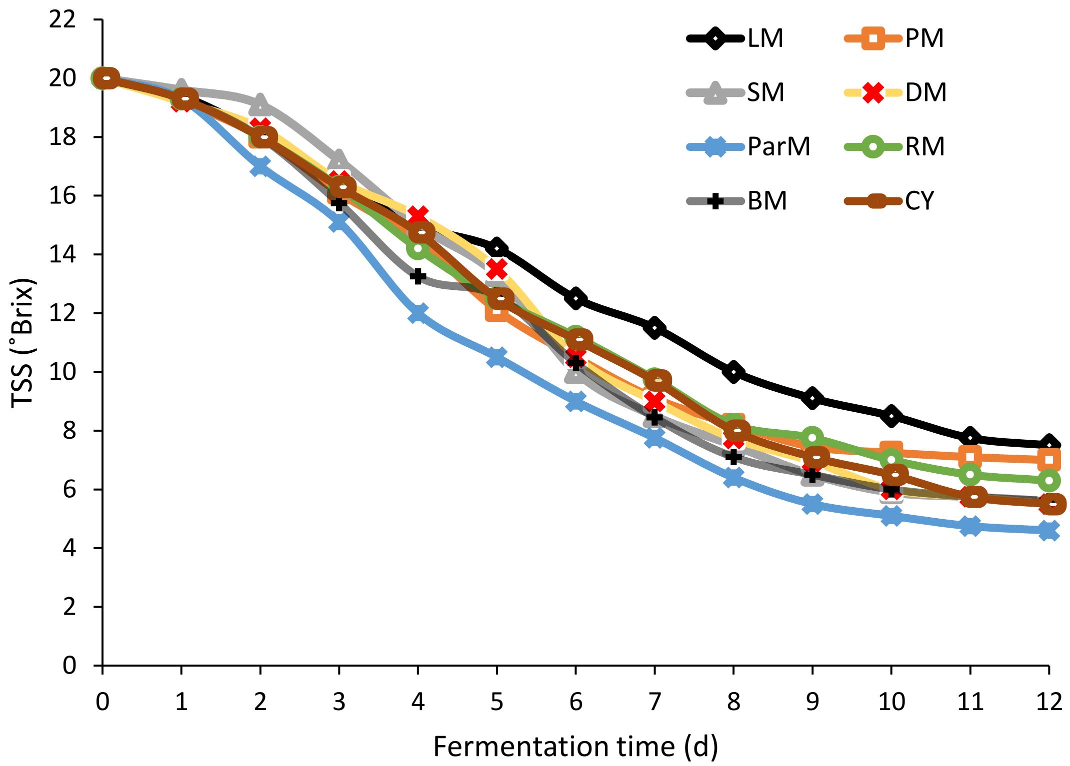


Fig. 4.11 Changes in total soluble solid (˚Brix) during fermentation in YEPD broth at 28˚C for 12 days.

Note: LM= Lalitpur *murcha*; PM= Palpa *murcha*; SM= Sunsari *murcha*; DM= Dolakha *murcha*; ParM= Parbat *murcha*; RM= Ramechhap *murcha*, BM= Bardia *murcha*; CY= commercial yeast

4.9.2 By dichromate oxidase method

This method of ethanol production test generally used for low concentration of ethanol production. The yeast isolates were examined for their alcohol production on YPD broth. Seven isolates were selected from different *murcha* samples based on their ability to form colonies on YPD agar plates containing 20% glucose.

Table 4.6: Concentration of ethanol of different yeast isolates

Code of yeast samples	Ethanol content (mg/ml)
LM	9.27
PM	5.95
SM	7.70
DM	10.53
ParM	9.87
RM	8.41
BM	7.32
CY	9.48

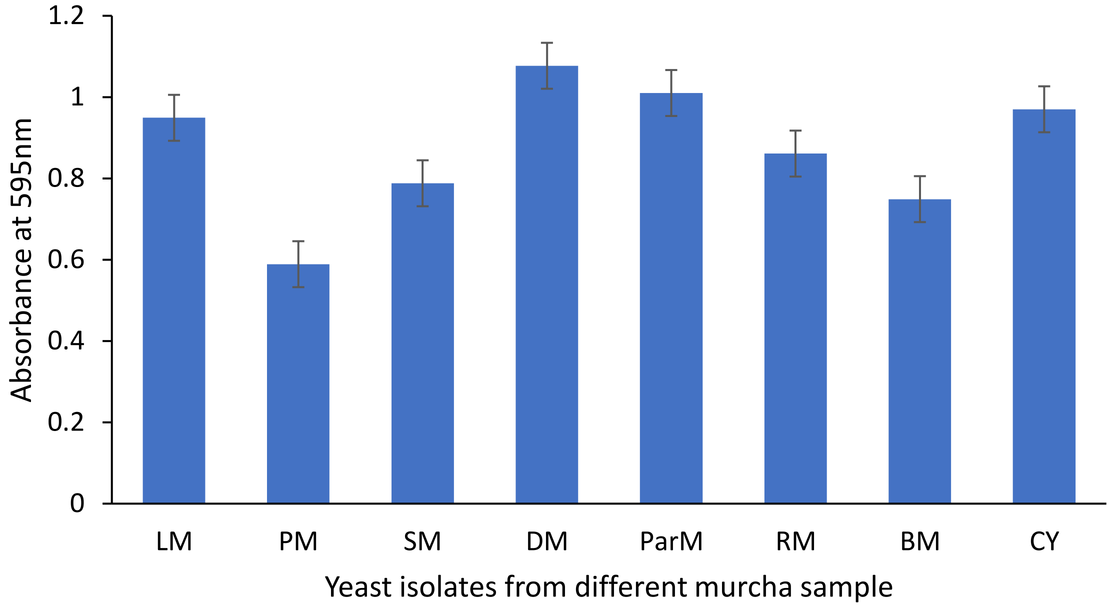


Fig. 4.12 Comparative ethanol production in YPD broth at 28˚C for 18 h and growth was observed O.D. at 595nm. Vertical bar indicates ± standard error n=2.

Note: LM= Lalitpur *murcha*; PM= Palpa *murcha*; SM= Sunsari *murcha*; DM= Dolakha *murcha*; ParM= Parbat *murcha*; RM= Ramechhap *murcha*, BM= Bardia *murcha*; CY= commercial yeast

The ethanol production was determined after 18 h of incubation at 28˚C. Dolakha yeast isolate showed the highest ethanol production (10.53 µg/mL) whereas all the other yeast isolates showed lower ethanol production (5 to 9 µg/mL) of ethanol including commercial yeast (Table 4.6). Ethanol production was calculated by using standard graph of ethanol (Appendix, Photo 2).

4.9.3 CO_2 production test

Mold and yeast isolates isolated from different *murcha* sample were incubated in conical flask containing steam rice (50 g) for 4 days at 28˚C and capped with small sized ballon to check carbondioxide production. All the isolates showed positive result to the alcohol and carbondioxide production (Fig. 4.13). This was subjective observation of ethanol production test by yeasts and mold isolated from different murcha samples.

Fig.4.13 Carbondioxide test from different yeast and mold isolates.

4.10 Saccharification test/selection criteria of mold

Saccharification test of mold isolated from different marcha sample was performed by liquestion test and estimation of sugar by dinitrosalicyclic acid (DNS) test.

4.10.1 Liquefication test

Mold isolates isolated from different *murcha* were incubated in conical flask containing steam rice (50 g) for 4 days at 28˚C, plugged the conical flasks by cotton. All the isolates had positive result to the liquefication test. All isolates produced liquid in conical flask containing steam rice (Appendix Photo 5).

4.10.2 Estimation of fermentable sugar

Mold isolates isolated from different *murcha* were incubated in culture tube containing PBS broth for 24 h at 28˚C and reducing sugar production was determined. Isolate of Parbat mold showed highest reducing sugar production (417.08 µg/mL) among all the other isolates (283.3 to 395.8 µg/ml) shown in Table 4.7. Fermentable sugar was calculated by using standard graph of glucose (Appendix, Photo 1).

Table 4.7 Concentration of reducing sugar produced by different mold isolates

Code of *murcha*	Reducing sugar content (µg/mL)
LM	335.0
PM	387.8
SM	283.3
DM	296.7
ParM	417.1
RM	395.8
BM	299.2

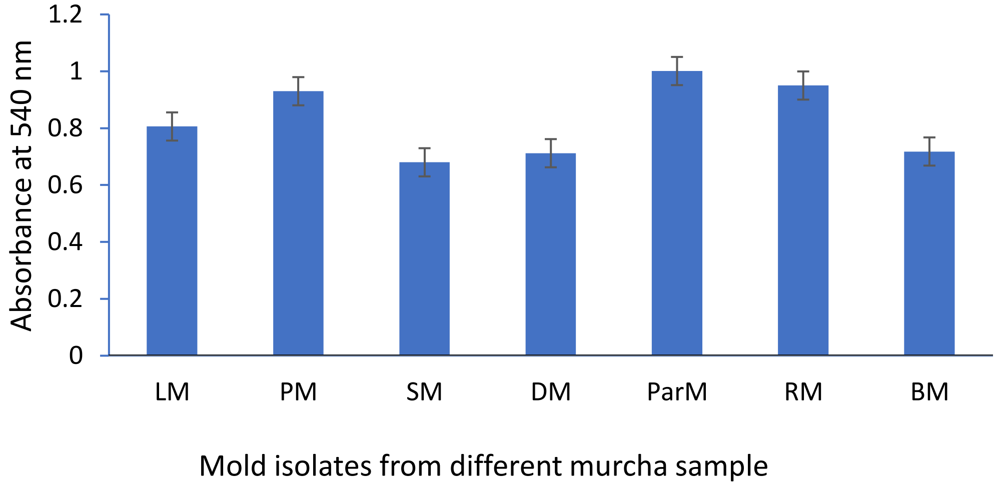


Fig. 4.14 Comparative glucose production in PBS broth at 28˚C for 24 h and growth was observed at 540 nm. Vertical bars indicate ± standard error n=2.

Note: LM= Lalitpur *murcha*; PM= Palpa *murcha*; SM= Sunsari *murcha*; DM= Dolakha *murcha*; ParM= Parbat *murcha*; RM= Ramechhap *murcha*, BM= Bardia *murcha*

4.11 Rice wine production from selected yeast and mold isolate

The inoculum for fermentation were prepared in YEPD broth and PBS broth of yeast and mold of Parbat *murcha*. Mold isolate was used to produce *koji-mold* for rice wine production. Fermentation was done in jar up to 13 days in duplicate at about (24±2˚C) obtained cloudy white liquid with fruity smell. Filtration through cheese cloth was done in 5 L flask, after chilling for 5 days. The filtrate thus produced was cloudy yellowish color. First locally available charcoal (*agar*) was used to clarify the fermented mash but it has little effect on clarification. Then activated charcoal was used. There was significant decrease in turbidity after passing through the activated charcoal (1200 IV).

By using locally available coal, there was no efficient filtration through coal packed material. Using activated charcoal instead of coal, there was significant decrease in turbidity and finally, white liquid *sake* obtained through filtration from activated charcoal (Appendix, Photo).

4.11.1 Changes in ˚Brix and pH during fermentation

Changes in TSS (˚Brix) and pH during fermentation of rice wine by using Parbat yeast and mold isolated from Parbat *murcha* upto 14 days of fermentation in room temperature was obtained as shown in Fig. 4.16. Continuous increase in ˚Brix of rice mash from upto day 4 and after day 4. And decrease in ˚Brix. After day 5 of fermentation there was no change in ˚Brix. And there was continuous decrease in pH during fermentation of *sake* from day 1 to day 5 at room temperature after day 5 there was no change in pH.

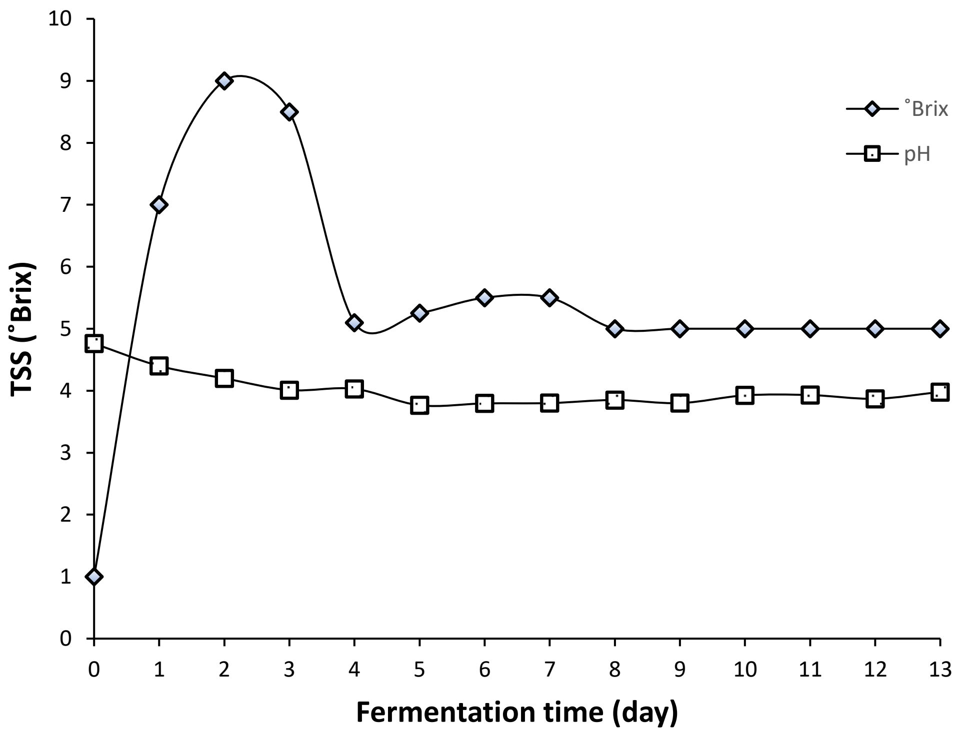


Fig. 4.16 Changes in ˚Brix and pH during fermentation of rice mash at room temp. (24±2˚C).

4.11.2 Changes in acidity during fermentation

Similarly, change in acidity in term of succinic acid during fermentation of rice wine by using

Parbat yeast and mold isolated from Parbat *murcha* upto 14 days of fermentation in room temperature was obtained as shown in Fig. 4.17.

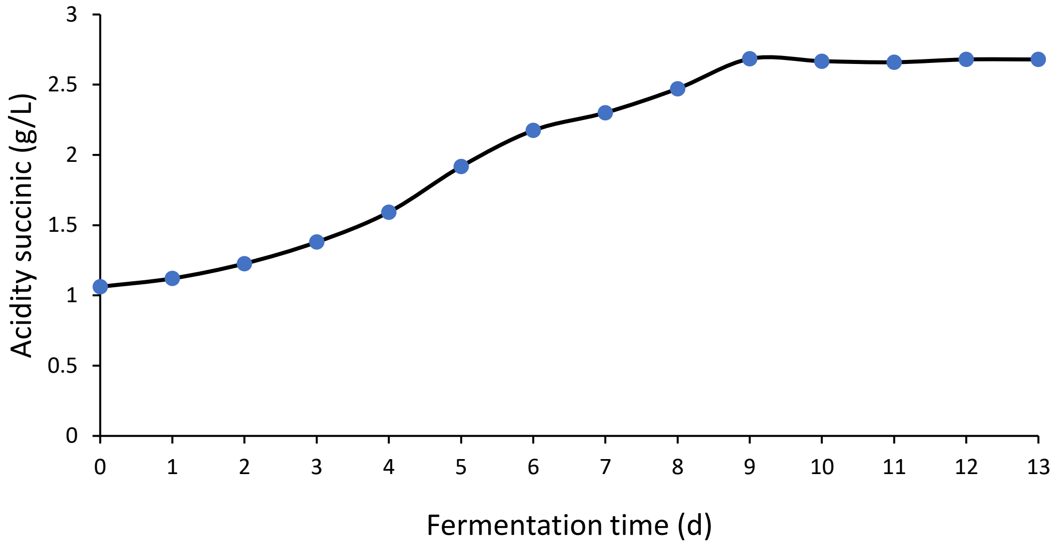


Fig 4.17 Change in acidity during fermentation of rice mash at room temperature(24±2˚C).

From day 1 there were continuous increase in acidity upto day 9 of fermentation after day 9 there was no change in acidity.

4.12 Comparative study of Lab prepared *sake*

Lab prepared sake and commercial sake were compared for their physico-chemical properties (Table 4.8).

Table 4.8 Comparative study of the lab prepared *sake* with commercial *sake*

S.N.	Particular	Non-pasteurized lab *sake*	Pasteurized lab *sake*	Commercial *sake*	*Sake*∗
1.	Alcohol (%abv)	5	5	18	13-17
2.	pH	4.65	4.7	4.1	4.2-4.7
3.	Succinic acid (g/L)	1.3	1.5	1.1	0.2-0.5
4.	˚Brix	6	5.8	9.5	6.3-11.3
5.	Glucose (g/100 mL)	0.60	0.56	2.5	0.5-2.4
6.	Glycine (g/L)	0.34	0.27	0.27	n.f
7.	Methanol (g/100 L abs. alc.)	_	_	_	n.f

∗standard values of sake given by Japan *sake* and *sochu* maker association

- indicates not detected, n.f indicates not found

CHAPTER 5
DISCUSSION

Scientist are continuously searching newer style of alcoholic products obtained through identification of noble type starter culture, modification of substrate and maturation technique to fulfil the consumer demand. Traditional starter culture (*murcha*) as used in Nepali *jand* and *raksi* preparation may be potential sources of yeasts and molds. The detailed of experimental works is described below:

5.1 Enumeration of yeast in different *murcha* sample

Murcha is the indigenous starter culture for the preparation of jand and raksi. It is a source of mold, yeast and bacteria (Dahal et al., 2007). In this work, different fermentative yeasts were isolated from *murcha* sample from seven different places (Table 4.1). *Murcha* sample collected from different places were round, oval and murcha from Bardia was flat shape. *Murcha* collected from different places were colored differently due to different flour (such as wheat, corn, millet, rice etc) used in its preparation. They were diluted to 10^{-10} to reduce the yeast load. The maximum number of the yeast's colonies were obtained from the *murcha* sample prepared from rice flour collected from Parbat yeast isolate. It was white in color and marketed for the preparation of *Jand.*

5.2 Identification

Yeast colonies were isolated from the YEPDA based on their ability to grow in the YEPDA, showing the characteristic of fungi. They were further isolated based on their color and texture. Gram's staining was performed to observe the shape and size of yeasts. All the yeasts isolates were oval or round with some slightly pointed to the top. Budding of yeasts were clearly observed in microscopic examination (Photo 8). The isolated strains grown on YEPDA media were found smooth surface with circular margin with creamy or creamy white and yellow white color similar to result reported by Yadav and Tiwari, 2016. Similarly, mold were isolated from the PBSA media based on their ability to grow on it. They were further isolated based on morphological character. All of mold isolates showed filamentous and mucoid with hard surface. Staining of mold isolates showed Sporangiophore with sporangium (Photo 7). Due to morphological character and starch hydrolysis test on PBSA media, the fungus was considered as *Rhizopus* (Photo 10).

Yeasts colonies isolated from YEPDA media grow on urease broth at 28˚C. In urea hydrolysis test, isolates showed the negative results in 24 h of incubation as shown in Fig 4.1, Yadav & Tiwari (2016) also reported the similar result. All yeast isolates utilize glucose and sucrose as carbon source except Ramechhap yeast isolates. Ramechhap yeast isolates cannot utilize sucrose and none of them utilized starch correspond to the Koschwanez et al., 2011. Not all yeast can produce invertase. The mutant yeast that are known as opportunists, because they avoid the energetic costs of breaking down the sucrose themselves, and instead consume the glucose and fructose produced by other yeast (Koschwanez et al., 2011).

5.3 Selection criteria for yeast

In this study different criteria were used for selecting yeasts such as sugar, ethanol, pH tolerance tests.

5.3.1 Sugar tolerance test of yeast strains

All the eight yeast isolates showed their growth on YEPD broth at 28˚C for 1 day contain 30% dextrose (Fig. 4.3). Lower sugar concentration was found to enhance the growth of yeast cells, whereas the increase in sugar concentrations inhibited their growth of yeast cells (Hohmann, 2002). Despite the decrease in growth at higher sugar concentration, the yeast cells were found to tolerate up to 40% sugar concentration which was higher than the result reported by Osho, 2005. Exposure of yeasts to hyperosmotic environment provokes a positive turgor pressure resulting in a rapid efflux of intracellular water into the medium leading to cell dehydration and sodium specific inhibition of certain proteins (Pretorius, 2000). The cytoskeleton collapses due to the rapid efflux of water through the lipid bilayer resulting in the growth arrest of the cell. Cellular reprogramming or adaption is the major defence response under these conditions. Yeast respond by accumulating intracellular polyols particularly glycerol, trehalose, arabitol, sorbitol and compatible ions (Pretorius, 2000). Activity of glyceraldehyde 3-phosphate dehydrogenase (GPD) is known to be increased during sugar and salt stress leading to the increased formation of glycerol which is the major stress protectant. The activity of alcohodehydrogenase is found to be decrease in hyperosmotic condition (Thomé, 2007).

5.3.2 Ethanol tolerance test of yeast strains

Eight different yeast strains were found to exhibit remarkable higher ethanol tolerance. All the strains showed significant growth at up to 14% ethanol concentration (Fig. 4.4). And above this concentration, there was a significant decrease in growth as reported by Xue et al., 2008. Lower concentration of ethanol was found to favour the growth of yeast cells whereas higher concentration of ethanol was found to inhibit and hence decrease growth. The ability of yeast to tolerate and grow in the presence of higher concentration of sugar have also been reported to tolerate to higher concentration of ethanol (Pais et al., 2013). Yeast cells treated with higher concentrations of sugar are known to show increased accumulation of trehalose, an important storage compound and a stress protectant. Trehalose was reported to confer the growth under hyper osmotic conditions and also helps to withstand toxic concentration of ethanol (Divate et al., 2016). Ethanol toxicity is one of the most common stresses that yeast cells encounter during fermentation (Furukawa et al., 2004). At low concentration of ethanol, yeast cells decrease the cell volume and increasing thermal death whereas higher concentration of ethanol reduces cell vitality increases the cell death rate (Stanley, et al., 2010). Higher level of ethanol in the medium affects the integrity of membrane by inserting into the hydrophobic region by increasing the polarity of region, thereby weakening the hydrophobic barrier to the free exchange of polar molecules. High concentration of ethanol also decrease the fluidity of plasma membrane leading to dissipation of trans-membrane electrochemical potential and subsequently acidify the intracellular and vacuolar conditions. Ethanol at higher concentration has also been shown to denature protein and its dysfunction (Divate et al., 2016). Cells exposed to ethanol stress respond by increasing the expression of genes associated with energy yielding pathways such as glucose transport and metabolism while reducing the expression of genes associated with energy demanding anabolic processes such as growth, leading to an initial growth lag period (Stanley et al., 2010).

5.3.3 pH tolerance test of yeast strains

As shown in Fig. 4.4, all the isolated yeast strains grow on YEPD broth at 28˚C for 1 day contain pH range from 3-5.5. There was a change in growth at different pH values, a similar result reported by Liu et al., 2015. Acidic condition was found to favour the growth of yeasts whereas slightly acidic or near to neutral condition inhibits the growth of yeasts (Narendranath & Power, 2005). In general, low initial pH shows the properties of prolonging yeast lag phase, resulting less accumulation of biomass mass, reducing the consumption rate of total sugar, increasing final content of acetic acid and glycerol, and decreasing final content of ethanol and L- succinic acid, except some special cases such as growth condition (Liu et al., 2015). External environmental pH especially caused by weak acid can affect the cell wall structure and alter the conformation of proteins protruding from the plasma membrane (Liu et al., 2015). It also has an impact on the lipid organization and function of cellular membranes and the perturbation of the function of membrane imbedded proteins. The loss of plasma membrane integrity increases cell permeability to ions and other small metabolites, which leads to the stimulation of passive diffusion of protons from the exterior to the cytosol. In this case, the reduction of internal pH in weak acid challenged cells and the dissipation of the electrochemical potential maintained across this membrane are easily occurred. Eventually, pH value affects the growth and fermentation rate of yeast and influences the constitution of fermentation products (Liu et al., 2015).

5.4 Molecular characterization of two selected yeast strains

The two selected strains, strain DD-CDBT (Dolakha yeast) and EE-CDBT (Parbat yeast) were sent for the sequencing and both found 99% identical to *Saccharomyces cerevisiae* and *Saccharomyces boulardii*. Blast result of Dolakha yeasts shown in Fig. 4.7. Similarly blast result of Parbat yeast as shown in Fig. 4.9. Phylogenetic tree of Dolakha yeast as shown in Fig. 4.8. Phylogenetic tree of Parbat yeast as shown in Fig. 4.10.

Saccharomyces cerevisiae is known for its fermenting ability utilizing hexoses. Saccharomyces yeasts are favoured because of their excellent ethanol yield, tolerance of low pH that discourage the growth of spoilage microbes. It has the ability to grow aerobically for efficient cell generation (Castilleja et al., 2017). The optimized condition for *S. cerevisiae* was found to be; pH 4.5, peptone as nitrogen source, 20% glucose condition and temperature of 28˚C. In this study *S. cerevisiae* found to produce ethanol 10.52 mg/mL. The metabolism of *S. cerevisiae* is programmed to catabolize external sugar's rapidly to ethanol, irrespective of presence of oxygen (Yang et al., 2018). It is thought that this behaviour provides a competitive advantage: *Saccharomyces cerevisiae* is tolerant upto 20% ethanol, while most competing yeast and bacteria are killed at more than 5% ethanol (Yang et al., 2018). This behaviour of yeast is important for sake preparation. pH is also key factor that affects growth of yeast and finally in ethanol fermentation as ability of ethanol production ability changes with pH. Below pH 4, the incubation time for maximum ethanol production prolongs and above pH 6, formation of other by-products such as acetic acid and butyric acid dominates. The pH value of 4.0-5.0 is thus regarded as operational limit for ethanol production (Lin & Tanaka, 2006). The optimum pH in this study was found to be 4.5 for DD-CDBT, which corresponds to Lin et al., 2012. Nitrogen is essential for yeast metabolism and growth and considered to be a limiting factor during fermentation process. The nitrogen sources not only increases biomass, but also reduce time required for the completion of fermentation (Karthikeyan & Kanchana, 2014).

Optimized condition for *S. boulardii* found to pH 4, peptone as nitrogen source, 20% glucose condition and temperature of 28˚C. In this study *S. boulardii* found to produce maximum ethanol 9.72 mg/mL. *S. boulardii* possesses unique abilities which allow it to survive in the most extreme conditions (Brandão et al., 2014). *S. boulardii* also has been known to secrete a variety of proteases which have inhibiting abilities on pro-inflammatory cytokines. *S. boulardii* is classified as an anaerobe, meaning it can grow under aerobic or anaerobic conditions (Dickinson, 1998). *S. boulardii* prefers fermentation over respiration 98 to 2% (Dickinson, 1998). Both the yeast strains showed maximum growth at 20% sugar concentrations. The combination of sugar tolerance and ethanol tolerance is an advantage when yeast is being considered for industrial ethanol production where a large amount of initial sugar being used (Ekunsanmi & Odunfa, 1990). The optimal sugar concentration of 20% for strains conferring ethanol tolerance of between 14-18% is like that of Osho, 2005. Finally got an accession number from GenBank, MK253787 and MK253281 for yeast isolates isolated from Dolakha and Parbat *murcha*.

5.5 Alcohol production test of different yeast isolates in YEPD broth medium

By hand refractometer all yeast isolates showed gradual decrease in TSS upto12 days. Day 1 showed very less decrease in TSS after day 1, there was continuous decrease in TSS up to day 9. After day 9 there was no change in TSS (Fig. 4.11), which was simiar result reported by Thammasittirong et al., 2013. The TSS (4.75˚Brix) was low and TSS (7.5˚Brix) was high during fermentation in the YEPDbroth fermented by Parbat yeast isolates and Lalitpur yeast isolates respectively. This could be since yeast isolates vary in their fermentation efficiency and sugar conversion ability. The continuous decreased in TSS in the initial period of fermentation may be due to faster conversion of sugars into alcohol which is available in maximum amount in the beginning of fermentation. Faster rate of fermentation at initial period may also be due to low alcohol levels in the beginning of fermentation. The fermentation rate declines later due to increased quantity of alcohol exerting effect on fermentation process by hindering the activity of yeast (Bhatane & Pawar, 2013).

By using hand refractometer showed there was no significant decrease in TSS in first day of fermentation but by using dichromate oxidase method showed that there was production of ethanol by all yeast isolates in 18 h (Fig. 4.12). Among them yeast isolated from Dolakha murcha showed highest production of ethanol (10.52 mg/mL). Ethanol production start from very first day of fermentation reported by Yadav &. Tiwari, 2016.

5.6 Saccharification test of different mold isolates

As shown in Fig. 4.1**,** all isolates of mold showed halo zone in the PBSA media. Maximum halo zone was showed by mold isolated from Parbat murcha. All the isolated mold were found to exhibit liquification. By DNS test also showed that there was significant increase in fermentable sugar in first day of incubation. Reducing sugar produced during fermentation at 28˚C for 24 h was (0.27-0.41) mg/mL (Table 4.6), which was less to result reported by Mathew, et al., 2016. The enzyme activity is depending on the time, pH, temperature and source of enzyme. In general enzymes are less stable at high temperature over time at pH value near the limit of the optimum. The optimum pH should be determined to be under certain conditions. In such case it is important to choose an enzyme with a pH range from 4 to 11 (Mathew, et al., 2016).

5.7 Rice wine production

During 13 days of fermentation of rice mash at room temperature (24±2˚C). change in ˚Brix, acidity and pH were calculated.

5.7.1 Changes in ˚Brix and pH during fermentation

As shown in Fig. 4.14 there was continuous increase in ˚Brix of wine from initial day of fermentation up to day 4 and decrease to day 5. After day 5 there were no change in ˚Brix. Continuous increase in ˚Brix may be continuous inoculation of yeast, *mold* and steam rice in fermentation tank during day 1, day 3 and day 4 of fermentation and also less activity of yeast strain to produce ethanol as compared to fermentable sugar produce by mold. After day 5 (ca 5˚Brix) of fermentation there were almost ceased fermentation that might be ethanol concentration or very less power of saccharification by mold. Chay et.al (2017) reported that finally change in ˚Brix stop at 5 ˚Brix during rice wine preparation.

Initial pH of fermentative mash was 4.5 so there was no addition of any acid to maintain pH of *sake* that might be acidic nature of koji, caused by growth of LAB during preparation and storage of *koji.* There was decrease in pH up to day 4 of fermentation (Fig. 4.14) that might be fermentation caused the production of ethanol and carbon dioxide. CO_2 is one of factor that decline pH of fermentative broth and production of organic acid such as succinic acid, pyruvic acid (Chay et al., 2017). After day 9 of fermentation there was slightly increase in pH, that was autophagy nature of yeast cell (Chen & Xu, 2013). Change in pH from 4.75 to 3.7 upto last day of fermentation, which showed similar result during prepataion of rice wine reported by Chen & Xu, 2013.

5.7.2 Changes in acidity during fermentation

There was increase acidity after day 1 of fermentation upto day 9 (Fig. 4.15) there may be formation of different organic acid and CO_2 formation during fermentation and after day 9 there was no change in acidity. And decrease in acidity might be decrease in fermentation. During initial and mid period of fermentation process there was a huge CO_2 evolution and this excess of CO_2 resulting in the formation of carbonic ions in the must and ultimately result in increased acidity. Whereas, at the end the titratable acidity decreased in all the treatments because of less CO_2 evolution and which resulted in lower carbonic ions in the must and thus low titratable acidity, besides production of some other organic acid during fermentation (Sharma et al., 2015). Bhatane & Pawar, 2013 suggested that glucose released from starch was almost completely converted to ethanol by the *sake* yeasts, whereas the glucose was partially used in other aspects of metabolism by laboratory yeasts. Titrable acidity in term of succinic acid produced during the fermentation of *sake* showed that continuous increased in pH from day 1 (1.062 g/L) to day 9 (2.98 g/L) after day 9 of fermentation there was no change in acidity (Fig. 4.16).

5.8 Comparative study of lab prepared *sake*

Sake production by using yeast and *mold* isolated from Parbat *murcha* after 13 days of fermentation at room temperature (24±2˚C). There was no addition of acid to adjust pH, due to acidic environment of fermentative mash that might be contamination of *koji* during preparation. But during *sake* preparation there is used lactic acid or LAB in Japan. *koji* was made from *mold* isolated from Parbat murcha (Fig. 3.8). As shown in Table 4.8 different chemical parameter were calculated. But ethanol (%abv) and ˚Brix were not within the value of commercial *sake*. That may be following reasons:

1. Generally *murcha* contain *Rhizopus*, so during *mold* preparation *Rhizopus* was used instead of *Aspergillus, Aspergillus* is used sake koji prepration in Japan.
2. Rice variety differ from *sake* rice.
3. Less polishing of rice.
4. Fluctuation of fermentation temperature.

The enzyme-producing properties (glucoamylase, α-amylase and protease) of *Rhizopus oryzae* found to better glucoamylase producer than *Apergillus oryzae* but in case of alpha amylase and protease, very less than *Aspergillus oryzae*. However, some fungal isolates of the same species exhibited a significant variability in the production levels for all determined enzyme activity (Lv, et al., 2012).

Rice wine qualities differs with rice varieties and fermentation methods and significant differences are observed with respect to various parameters like pH, titratable acidity, total soluble solids, alcohol contents, amino-N, protein and reducing sugar and overall sensory acceptance (Chay et al., 2017). The highest ethanol concentration and glycerol concentration both are obtained at the fermentation mash treated at 23°C. The highest peak value of maltose (90 g/L) is obtained at 18°C. Lactic acid and acetic acid both achieved maximum values at 33°C. Temperature contribute significantly to the ethanol production, acid flavor contents, and sugar contents in the fermentation broth of the Chinese rice wines (Liu et al., 2014).

Rice wine made using rice polished at 70 and 60% showed higher rates of fermentation, higher alcohol and sugar contents than rice wine produced using rice polished at 90 and 80%. Based on the proximate composition of rice and physico-chemical properties of rice wine, rice polished at 60 and 70% was found to be better than rice polished at 80% and 90% for rice wine production (Arachchige et al., n.d.).

CHAPTER 6
SUMMERY

In this study, seven different isolates of yeast and mold isolated and preserved were used for determining their sugar pressure, ethanol tolerance, pH tolerance and alcohol production test by comparing yeast isolates with commercial yeast strain (*Saccharomyces bayanus* SN9). Among the seven different yeast strains, Parbat yeast showed maximum sugar tolerance of 40%, highest ethanol tolerance of 15% along with maximum ethanol production and maximum pH tolerance range 3-5.5. Dolakha yeast showed maximum ethanol production in very first day of fermentation. Maximum liquification also showed by Parbat mold isolated from Parbat murcha. And maximum CO_2 production were observed fermented mash of steam rice by inoculation of yeast strain and mold strain isolated from murcha sample collected from Parbat and Dolakah. Yeast and *mold* isolated from *murcha* sample collected from Parbat were selected for *sake* production in pilot scale. These two selected strains were further identified by sequencing. Both strains DD-CDBT(DY) and EE-CDBT(ParY) were confirmed to be *Saccharomyces cerevisiae* and *Saccharomyces boulardii* respectively. After submission of sequence in GeneBank, got an accession number from GenBank, MK253787 and MK253281 for yeast isolates isolated from Dolakha and Parbat *murcha*.

The *sake* produced by strain isolated from Parbat *murcha* (PM) under condition was found to be 5% ABV, acidity in term of succinic acid (g/L); 1.5 and 1.3, amino acid in term of glycine (g/L); 0.27 and 0.34, glucose (g/100ml); 0.56 and 0.6, ˚Brix; 5.8 and 6 for pasteurized and non-pasteurized *sake* respectively. It was compared with commercial *sake* based on physico-chemical properties. The lab *sake* had similar quality parameter except alcohol content and ˚Brix. Commercial *sake* had 18% abv and 9.5 ˚Brix as compared to 5% abv and 6 ˚Brix in lab prepared *sake*.

CHAPTER 7
CONCLUSION AND RECOMMEDATION

7.1 Conclusions

The following conclusions were drawn from this study:

1. *Murcha* sample collected from Parbat had significant tolerance of alcohol, sugar, pH and highest alcohol production as well as liquification of steam rice among all murcha sample including commercial yeast (*Saccharomyces bayanus* SN9).

2. In *murcha* sample there was generally presence of *Rhizopus* species that had less capacity of saccharification of starch.

3. During alcoholic fermentation change in % of alcohol affect the different chemical composition of final product.

4. Filtration through activated charcoal was little changes in flavour but acidity in term of succinic acid is greatly affects *sake* product.

5. Low calorie *sake* can be produced by using yeast and mold isolated from Parbat *murcha* and local variety of rice *(taichin).*

6. There was no significant different of physio-chemical effect of pasteurized and non-pasteurized sake within short period of storage.

7.2 Recommendations

Since ethanol produced less amount during the fermentation of rice wine by using yeast and mold isolated from local starter culture (*murcha*). Hence, for the further work, following suggestions are recommended.

1. Sake production by using *Rhizopus* isolated from *murcha* was found to be less saccharifying potential as compared to *Aspergillus oryzae,* for producing high alcohol rice wine. *Aspergillus oryzae* can be used.

2. Different varieties of rice can be fermented to evaluate the impact of rice quality on the final product.

3. Polishing ratio of rice can be increased to obtain to obtain more alcohol content and cleaner flavor.

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APPENDIX

Appendix 1

YEPD Agar	Ingredients (g/L)
Yeast extracts	3g
Peptone	5g
Glucose	10g
Agar	20g
Distilled water	1L

Appendix 2

PBS Agar	Ingredients (g/L)
Peptone	5g
Beef extract	3g
Starch	2g
Agar	15g
Distilled water	1L

Appendix 3

DNA lysis buffer	Composition
Titron X-100	2%
Sodium Dodecyl Sulphate (SDS)	2%
Sodium chloride (NaCl)	0.1M
Ethylene-diamine-tetra-acetic acid (EDTA)	1mM
Tris – HCl pH 8	0mM

Appendix 4

Tris EDTA buffer	Composition
Tris pH 8	1mM
EDTA pH 8	10mM

Appendix 5

50 x Tris Acetate – EDTA (TAE) buffer	Composition
Tris base	24.2 gram
Glacial acetic acid	5.7 ml
0.5 M EDTA (pH 8.0)	10 ml

Final volume 100 ml to be made with Distilled Water

Appendix 6

Urea test broth	Composition (g/L)
Urea	20
Na_2HPO_4	9.5
KH_2PO_4	9.1
Yeast extract	0.1
Penol red	0.01

The pH is made to 6.8±0.2 at 25˚C.

Photo gallery

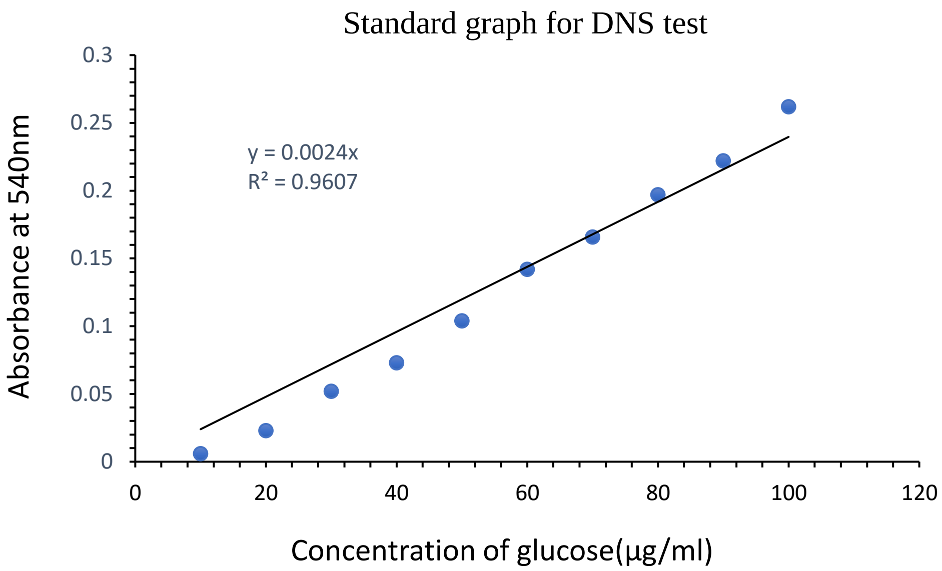

Photo 1 Standard graph for DNS test and concentration of glucose of different mold

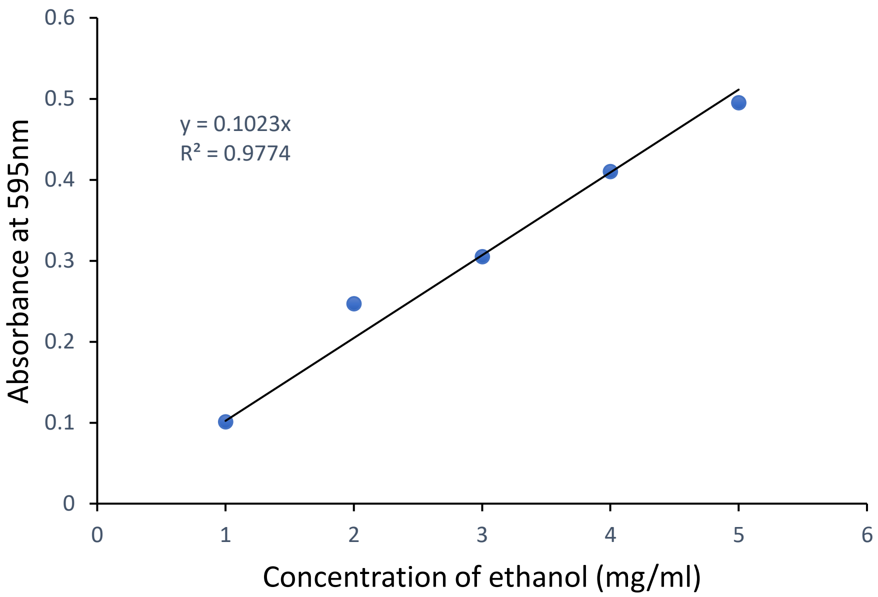

Photo 2 Standard graph for ethanol estimation

Photo 3 *Murcha* **Photo 4** Paddy and polish rice

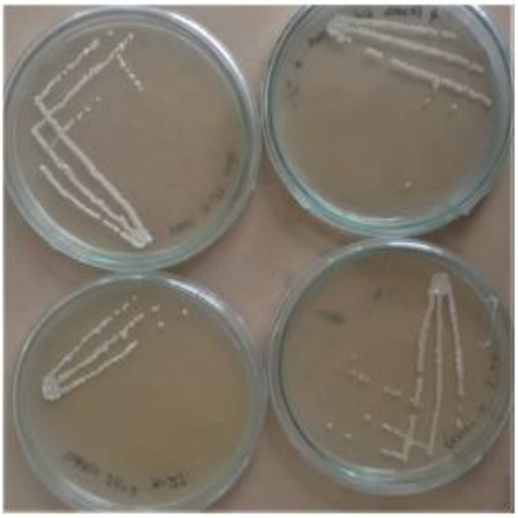

Photo 5 colonies of yeast on YEPDA plates

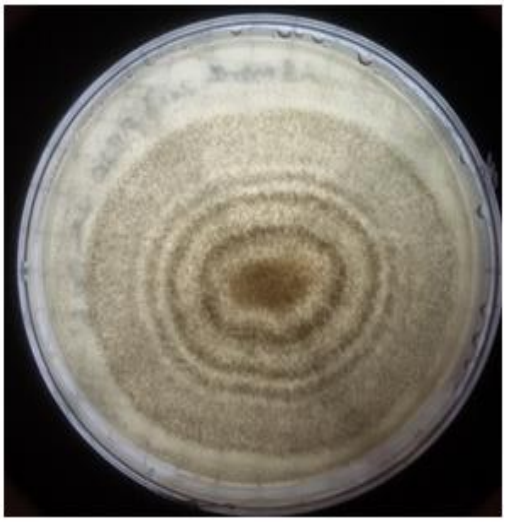

Photo 6 Mold growth appeared on PBSA plate

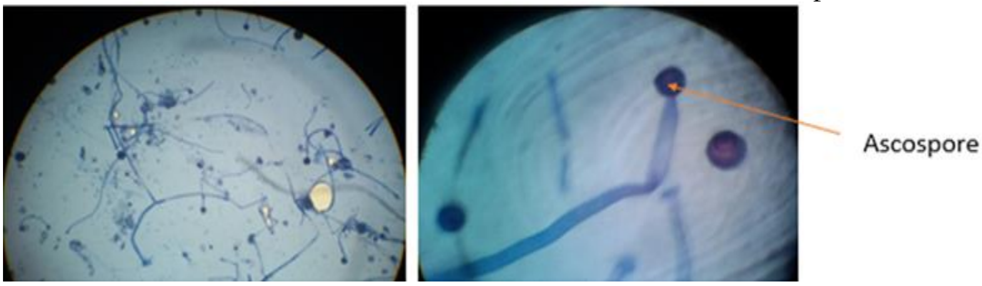

Photo 7 microscopic observation of mold

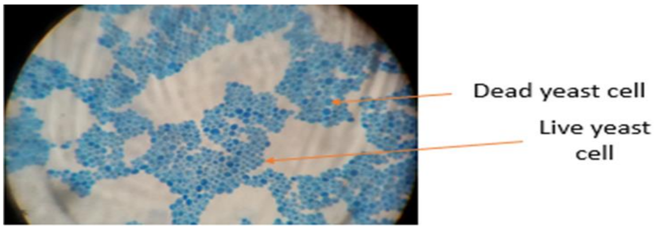

Photo 8 Microscopic observation of yeast cells

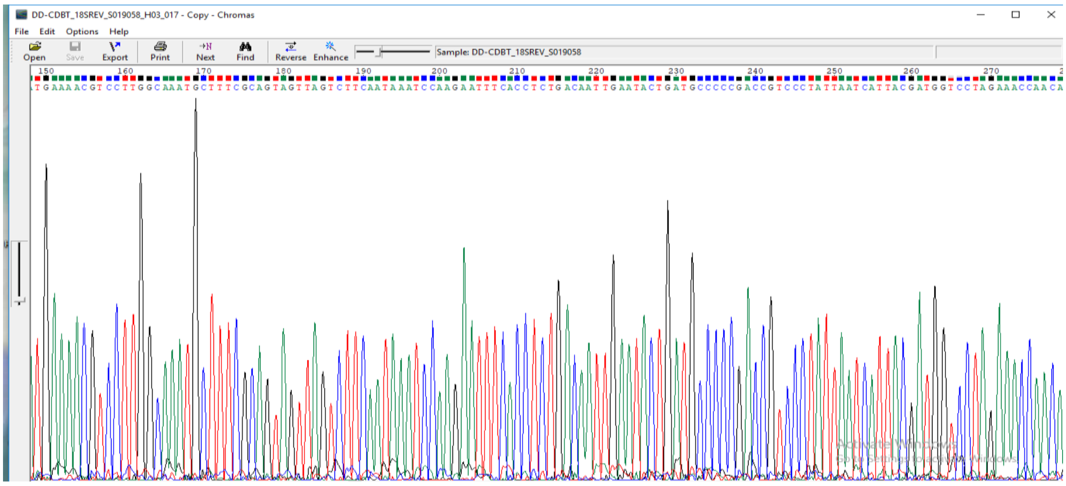

Photo 9 Chromatograph sequence of yeast isolate from Dolakha yeast

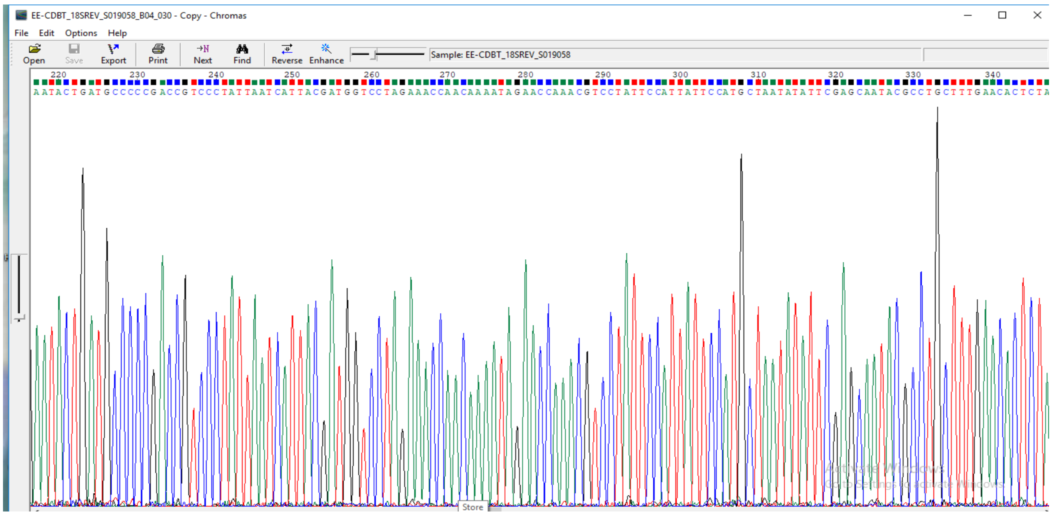

Photo 10 Chromatograph sequence of yeast isolate from Parbat murcha

Photo 11 *Koji-mold*

Photo 12 Filtration of fermentative mash through cheese cloth

Photo 13 *Sake* mash after filtration

Photo 14 *Sake*, after cold treatment

Photo 15 *Sake* filtration through activated charcoal

Photo 16 *Sake*, after filtration through activated charcoal

Photo 17 *sake*