PRODUCTION OF *SAKE* FROM LOCAL VARIETY OF RICE USING ISOLATED MOLD FROM LOCAL STARTER CULTURE, *MURCHA*

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ACRONYMS

GC/MS	Gas Chromatography Mass Spectrometry
OD	Optical density
CYA	Czapek yeast agar
DNS	Di-nitro sulphonic acid
TSS	Total soluble sugar
LAB	Lactic acid bacteria
CMA	Coconut milk agar
YEPDA	Yeast extract potato dextrose agar
PBSA	Peptone Beef extract Sucrose Agar
Uv	Ultra violet
YES	Yeast extract sucrose
CFU	Colony forming unit
V/V	Volume/Volume
Abv	Alcohol By Volume
Mg/L	Milligram/litre

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ABSTRACT

Rice wine is alcoholic beverage made by simultaneous saccharification and fermentation by using mold and yeast. Rice (*Oryza sativa*) is staple food for half the world population. In Nepal, local starter culture (*Murcha)* has been used for starter culture for production of cereal based alcoholic beverage. The quality of alcoholic beverage always varies due to lack of process standardization in term of culture and process. There for 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. Seven molds isolates from *murcha* were tested for saccharification by halo zone on starch media, microscopic observation, liquefication and DNS test. All the isolated molds exhibit better growth in YPD media and showed positive result of starch hydrolysis. Among all the isolates the mold isolated from *murcha* (Rajbiraj) showed better saccharifying capacity than other isolates. It showed 36% saccharifyimg capacity, higher than that of other isolates. The mold which have higher saccharifying capacity is used for production of rice wine. In the rice wine total volume of alcohol was found to be 8%, pH was 3.42, succinic acid was 3.9 mg/L and ester was 11.1 mg/L. The lab prepared rice wine was compared with a commercial rice wine, and found comparable with respect to alcohol content, pH, total acidity and ester content. The essential oils was determined by using GC/MS.

Key words: *Murcha*, saccharification, fermentation, GC/MS, rice wine

CHAPTER 1

INTRODUCTION

1.1 Overview of rice

Rice (*Oryza sativa*) is staple food for half the world population. It is native grain of Southeast Asia, and one of the leading food crops of the world. Rice is predominantly an Asian crop, 95 per cent of it is being produced and consumed in the Southeast Asian countries. China and India alone account for 50% of the rice grown and consumed (Muthayya *et al.*, 2014). Rice is one of the most important food crops in Nepal. It is mostly grown in Terai region. It is major food crop in Nepal and contributes significantly to livelihood of majority of people and to the national economy.

Aspergillus oryzae is fungus widely used in production of traditional Japanese fermented food products. *Aspergillus oryzae* was first isolated from *koji* by H. Ahlburg in 1876, its original name was *Eurotium oryzae* later renamed as *A. oryzae* by F. Cohn, because it lacked the ability of sexual reproduction. It is filamentous fungus and has ability to produce various enzyme in large amount. *A. oryzae* is different from other filamentous fungus like *Mucor* and *Rhizopus*, because it produces protease which break down proteins (Machida et al., 2008).

Aspergillus is name is used for the genus of the molds which reproduce only by asexual means through conidiophore, the structure which bears asexual spores. The defining characteristic of the genus *Aspergillus* is the aspergillum-like spore-bearing structure. *A. oryzae* is aerobic filamentous fungus belongs to *Aspergillus flavus* group. This group contains industrially important species such as *A. oryzae* and *A. sojae* which are agronomically and medically significant fungi, they produce citric, gluconic, itaconic and kojic acid. Some species of this group are pathogenic to plant and animal. In agriculture aspergillus species are consider as an opportunistic pathogen of field crops. Afatoxins consider as important mycotoxin produce by *A. favus* and *A. parasiticus,* cause afatoxicosis in human (Bennett, 2010). Although *A. oryzae* is genetically very close to *A. flavus*, which is known to produce the most potent natural carcinogen, aflatoxin, *A. oryzae* has no record of producing aflatoxin or any other carcinogenic metabolites.

1.2 Fermented alcoholic beverages from rice

Varieties of alcoholic beverage based on fermented rice are highly common in different countries. It is an important indigenous fermented product, plays vital role in spiritual and cultural life in many indigenous tribes. While traditional alcoholic beverage made from rice have different composition, according to principle and manufacturing process can be characterized as biochemical modification of cereal starch. The microorganisms

(yeast and mold) play essential roles. Mold produce amylase which breaks down the cereal starch into dextrins and sugars and yeast convert sugar to alcohol (Dung, 2013).

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).

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 alcoholic drink produced from polished rice fermentation. In comparison to wine, in which sugar is converted to alcohol. However, in sake production is similar to that of beer, in which starch is converted to sugar and then fermented to alcohol. The brewing process for *sake* is different to beer. In beer, conversion from starch to sugar and from sugar to alcohol occurs in two distinct steps, but in sake conversion from starch to sugar and from sugar to alcohol occurs simultaneously. In addition, between wine, *sake* and beer alcohol content varies. Wine generally contains 9-16% abv, while most of beer contains 3-8% abv, and sake contains 18-20% abv (Issara & Rawdkuen, 2016).

Rice policing is first process in *sake* brewing. This approach includes removable of lipid, protein and minerals found mostly on outer part of rice grain. The polished rice is the washed and dipped in water and steamed for 30 to 60 minutes (Arendt & Zannini, 2013). The first fermentation stage is to produce seed mash. The water, yeast and koji are combined with steam rice, which is then fermented for 15 days at 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.

A turbid filtrate is usually obtained by charcoal filtration from the fermented mash through canvas bag. 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 & Rawdkuen, 2016).

1.2.2 *Makkoli*

Makkoli or *makgeolli* is rice wine, which means it is mostly produce using rice rather than grapes. It is popular Korean drink that dates back to the seventh century and has traditionally been cheap drink for poor. More recently it has become popular in Korean bars, with various brands of *makkoli* served on tap. It is made up of cooked sweet rice and *nuruk*, dry fermented cereal cake that acts as starter, encouraging mold growth which produce sugar that in turn produce alcohol. In addition to rice other grains, as wheat or barely, may be used for various characteristics. The mixture is left in clay pots to ferment for about a week.

Makkoli is unfiltered and naturally fermented, it is best drunk within the week or two after production. It will turn to rice vinegar within few months (Lee & Kim, 2019).

Makkoli is unfiltered Korean rice wine with cloudy and milky look. The low-alcohol wine is effervescent (lightly sparkling) and has a sweet-tart flavor profile. It is one of Korea's oldest alcoholic beverage, consider as farmer's drink and usually very cheap. Because of natural probiotics it has limited selflife. Usually it has mildly sweet taste but some cheaper varieties can be particularly sweet with added sugar or aspartame. It is slightly tangy, similar to yoghurt, since it is unfiltered. Thanks to natural fermentation, it has low to medium acidity and no tannins since it is not produced by using grapes.

Since *makkoli* is an unfiltered fermented drink, it contains healthy bacteria similar to yogurt. These bacteria can help in digestion, giving potential health benefits. It has low alcohol content as compared to other rice wines, making it less intoxicating (Imatome-Yun 2016).

1.2.3 *Soju* or *Shochu*

Soju is Korean most popular alcoholic beverage, it is also widely drunk in Japan and China. It is traditionally made from blended rice and other grains. It has been identified as being similar to vodka, since it is distilled. It is not because, soju has much lower in alcohol content (20 to 24% abv). *Soju* is clear in appearance with a clean taste, viscous texture and hints of sweetness on the finish, and blends well with whatever food is on the table (Ming, 2019).

1.3 Alcoholic beverages of Nepal

The history of alcoholic drinks in Nepal dates back to ancient times. These technologies were developed by ethics groups while celebrating various festivals, feast and marriage ceremony (Regmi, 2007). The concept of home brewing was pass down from generation to generation with little awareness of since and technology. Among different fermented food varieties jand, raksi, toongba, nigar and hyan thon are major alcoholic beverage, traditionally prepare and consumed in different parts of country. *Murcha* is necessary for the preparation of traditional alcoholic beverage (Rai, 2016).

In traditional method of cereal fermentation, all the process is followed as code of practice under unclear condition. *Murcha* is traditional starter culture for rice wine production. Different study shows that it contains both harmful and beneficial microorganisms, due to lack of quality control and technical knowledge of procedure. Its use has resulted in inconsistent quality of products. 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, 2013).

Although cereal based alcoholic beverage technology has been established in Nepal since ancient times, its production was limited to very 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 (Paudel & Thapa, 2015).

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

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 boost traditional Nepali food and beverage. The specified fermented starter for fermentation not only ensure the product's protection but also the necessary flora in the product (Paudel & Thapa, 2015).

Rice wine has been commonly sold in several countries like Japan, Korea, China, Malaysia and many Asian countries in wide scale. In Nepal household scale is produce for their won use. 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 wines are on demand monthly in the market. They have spent a lot of money on import raw materials from other countries. If locally available substrates are used for the production, it could help to reduce overflow huge amount of Nepali currency to abroad, thereby making benefit to both industry and farmers (Nepal Foreign Trade Statistics fiscal, 2020).

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 Objectives

The general objective of this study was to prepare rice wine using domestic rice variety, yeast, and mold from local sources.

The specific objectives were:

- Isolation and characterization of mold from *murcha* collected from different district of Nepal.
- To select the suitable mold for production of rice wine.

- Comparison of lab prepared rice wine with commercially available rice wine.

1.6 Scope

The production of stable fermentation starters will be attractive for small-scale food fermentation application. A good established fermentation starter, reduce fermentation time, minimize the loss of dry matter, prevent the growth of unwanted microorganism that cause off flavor in product, finally increase stability and success rate in production. In Nepal, there is huge possibility of producing alcoholic beverages from rice (a similar product of Japanese sake). It will not only minimize currency overflow, but also improve skills and technology, utilization of local raw materials, boost tourism and increase Nepali people's job opportunities. Nepal import 457,000 liters of alcohol worth Rs734.3 million similarly 153,000 liters of beer worth Rs 16.2 million and export 171,620 liters of alcohol worth Rs 35 million in 2019. There is big gap in import and export of alcoholic drinks, to minimize the importation of alcoholic beverage is industrially production of rice wine is one of the ways to reduce import of alcoholic beverages.

According to World Health Organization and Nepal Health Research Council, at least 66 per cent of alcohol consumed in Nepal either illegal or home-produced, making illicit market more than twice the size of legal market (Shakya, 2013). Spikes in demand for illicit alcohol also present severe health risks to consumers exposed to end product that do not comply with proper sanitary, quality and safety regulation, which are contaminated with toxic chemical additive. To prohibit the illicit alcohol a better approach would be Industrialization of home-produced alcohol. Which ensure the availability of legitimate products, enforce health and safety regulation in sector and collect proper tax revenues important to public investment in Nepal.

CHAPTER 2

LITERATURE REVIEW

2.1 Historical background of fermentation

Fermentation is one of the oldest techniques for preserving food. Indigenous fermented food such as beer, wine, cheese and mead (honey wine) have been produced and consumed for thousands of years, linked strongly to culture and tradition of the society especially in rural households and village communities. Indigenous fermented foods are classified according to different criteria. They may be classified according to the substrate used, the major type of fermentation process taking place; eg lactic acid fermentation, beverages, vinegar and bread. The type of cereals used in this process and the region of the indigenous for subclass of each category (Pires & Bránýik, 2015).

The term fermentation coms from Latin verb 'fervere' which means to boil. This term describes what happens when yeast converts fruit juice and malted grain converts into alcohol. There is reliable information that fermented drinks are produced over 7000 years ago in Babylon, 5000 years ago in Egypt, 4000 years ago in Mexico and 3500 years ago in Sudan (Hesseltine & Wang, 1972). Human beings are known to have made fermented foods since Neolithic times. The earliest types were beer, wine, and leavened bread (made primarily by yeasts) and cheeses (made by bacteria and molds). These were soon followed by East Asian fermented foods, yoghurt and other fermented milk products, pickles, sauerkraut, vinegar (soured wine), butter, and a host of traditional alcoholic beverages (Shurtleff & Aoyagi, 2007). Traditional fermented food plays an important role in East Asian food systems. These fermented foods have several important distinguishing characteristics; many important fermentation use molds; dairy products and other animal proteins (excepting fish) are not widely used, as they are in the West; and modern fermentation processes and technology are based largely on traditional processes, yet are extremely advanced and sophisticated. The main use of mold in the process of making *koji*, which serves as more than 50 enzymatic sources in the subsequent fermentation process. The form of *koji* was originated in China whereas the letter was developed in Japan about 1000 years ago. From the early of *koji* making process can be traced back to 300 BC in China and third century in Japan. Molds are different from bacteria and yeast in one important aspect that they can be easily observed with the naked eye and their growth, form and colour (Tamang *et al*., 2020). In early time until the 1870s, the traditional fermented food industries in East Asia were advanced largely by an empirical process without the general scientific research into the nature of microbes and process of fermentation and any theories in these areas (Waché *et al*., 2018).

2.2 History of *sake* brewing

Sake is a traditional Japanese alcoholic drink made from rice grain called *nihonsu* in Japan. *Sake* developed after rice was first cultivated in Japan over 2000 years ago, *Kuchikamizake* one of the earliest form of drink on record. At that time no machinery or technology required just, those with strong enough jaws and teeth who could chew grain of rice. This mouth chewed rice grain was spat into vat and enzyme in human saliva, along with natural yeast would produce alcohol in little amount. At that time the brewing process was quite rudimentary, whole of rice grain being used including brown outer parts. According to the record of ancient matters in the eighth century, a brewing department was established within the imperial place in Nara (Sato & Kohsaka, 2017).

In between 1478 to 1618, the record of daily temple life was written as Timon'in diary, which includes the process of policing or milling rice (to remove the brown outer covering to leave 100% white rice), the addition of the mixture in three-stage and form of pasteurization, in use 200 years earlier than Louis Pasture gave his name to the process in Europe. In 1578 the first time the sake has been filtered sufficiently to clear like water (Kanauchi, 2013).

2.3 Introduction of *sake*

Sake is an alcoholic beverage made up of rice and water through fermentation and filtration. It resembles white wine in appearance, ranging from transparent to slightly yellow (Nrib, 2013). *Sake*, a traditional fermented alcoholic beverage, is produced from polished rice (*Oryza sativa* Japonica sp.), rice mold (*Aspergillus oryzae*), and *sake* yeast (*Saccharomy cescerevisiae*) by simultaneous saccharification and fermentation. Rice mold produces large amount of starch hydrolyzing enzymes and protein breakdown enzymes. In addition, rice mold produces lipids, amino acids, vitamins, and secondary metabolites. *Sake* yeast, in turn, produces ethanol and several types of higher alcohols, acetic ester, ethyl esters, and many types of organic acids. *Sake* thus contains a variety of volatile and non-volatile metabolites derived from rice and microorganisms, which often differ depending on the brewing process and conditions, techniques employed, pasteurization process, and the storage conditions similar to that which occurs during winemaking and beer brewing processes (Takahashi & Kohno, 2016). *Sake* contains 13%-17% alcohol slightly higher than that of beer and wine, it also has a slightly 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).

Table 2.3 composition of sake, beer and wine

	Sake	Beer	Wine
Alcohol (% abv)	13-17	4-6	10-13
Glucose (g/100 mL)	0.5-4.2	0.003-0.1	0.1-3
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	40-120	500-1500
Malic acid (mg/L)	100-400	50-120	250-5000
Tartaric acid (mg/L			1500-4000

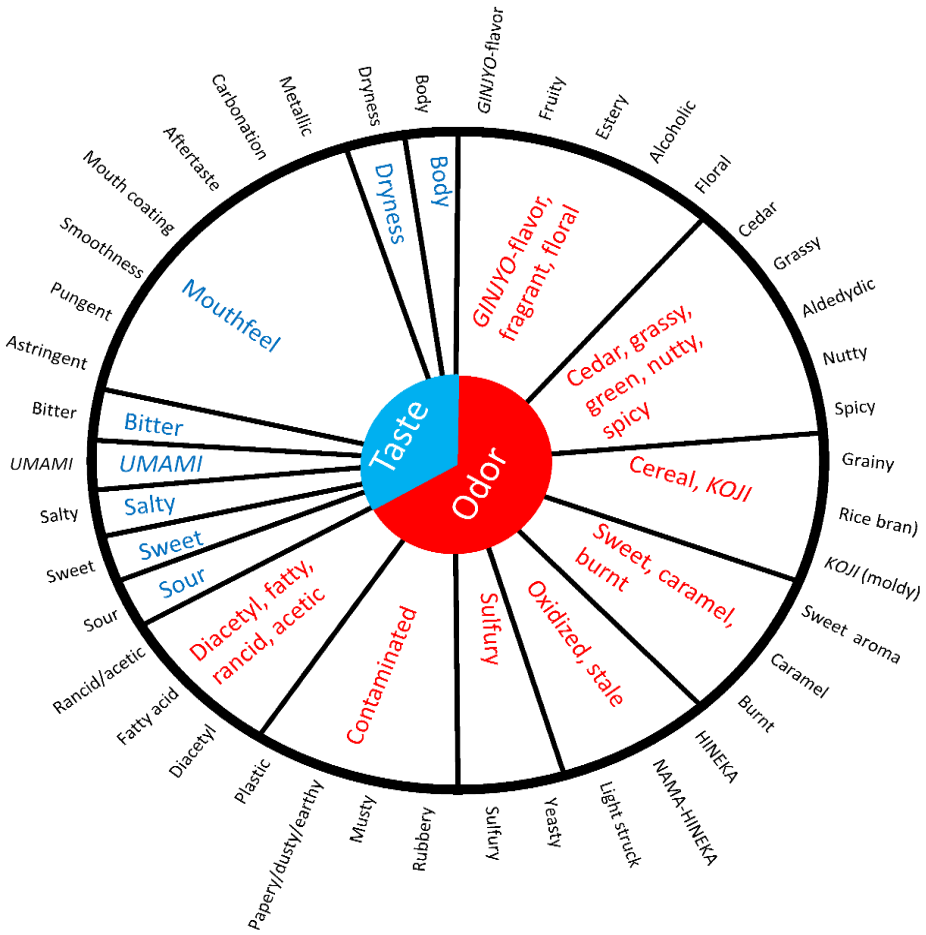

Figure 2.1 Flavor wheel of sake (Drahansky et al., 2016).

2.4 Outline of sake brewing

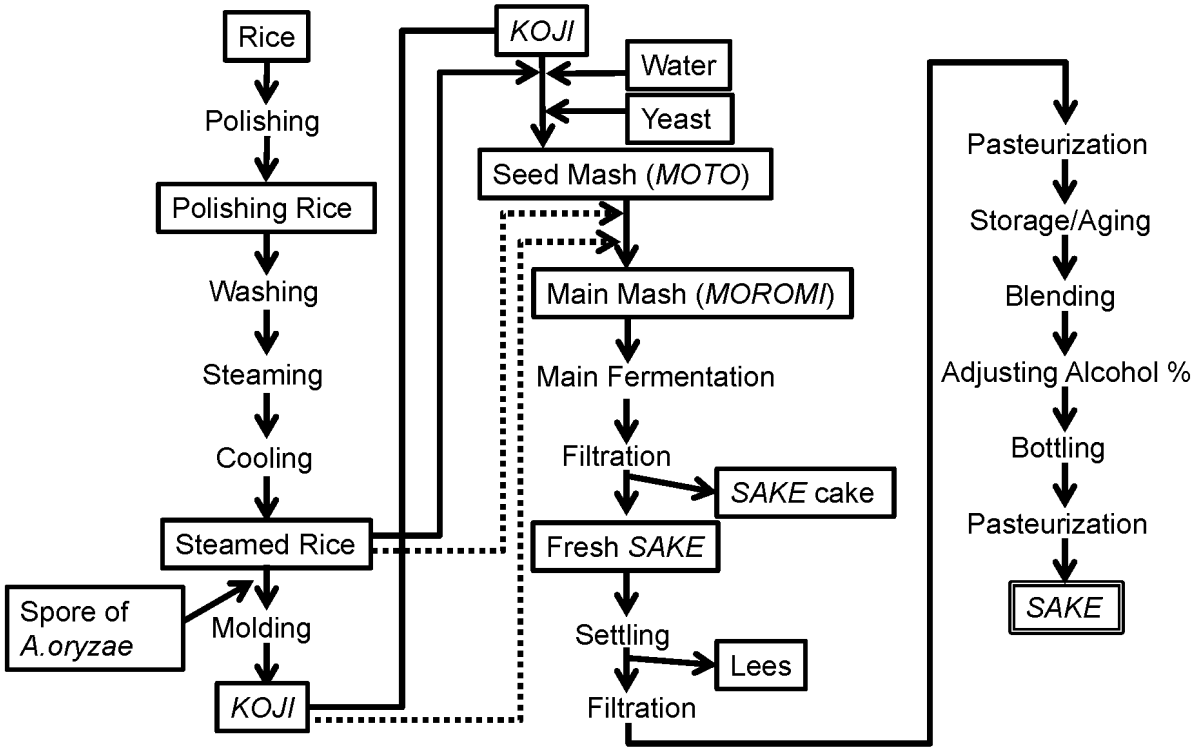

Figure 2.2 Outline of sake brewing (Drahansky *et al*., 2016)

2.5 Rice and rice polishing

One of the three main ingredients *sake* is rice. However, rice used for sake is different from the one we normally eat. There are mainly two varieties of rice; Indica, a long-grained variety, and japonica, a short grained variety. The long-grained with the large proportion of the inner part that is close to pure starch rice is mainly used for *sake* brewing. Each of these can further divided into sticky and non-sticky rice. Non-sticky japonica rice grown in Japan is used to brew s*ake*.

Other components such as proteins or lipids in rice, excepting starch, are unnecessary for *sake* production. In fact, *sake* produced with rice having excessive proteins or lipids does not have good flavor or taste. Their compounds exist on the endosperm surface, mainly around the aleuronemlayer. Therefore they are removed by rice polishing (Drahansky *et al.*, 2016).

The amount of polishing that is required in order to make one of the premium styles of sake is defined in Japanese law. This is called the polishing ratio. This ensures that the labelling terms are a good indication of the style of sake. The polishing ratio is expressed as a percentage. If the law requires a ratio of 60 per cent, this means that only 60 per cent or less of the original grain remains after polishing (Nrib,2013).

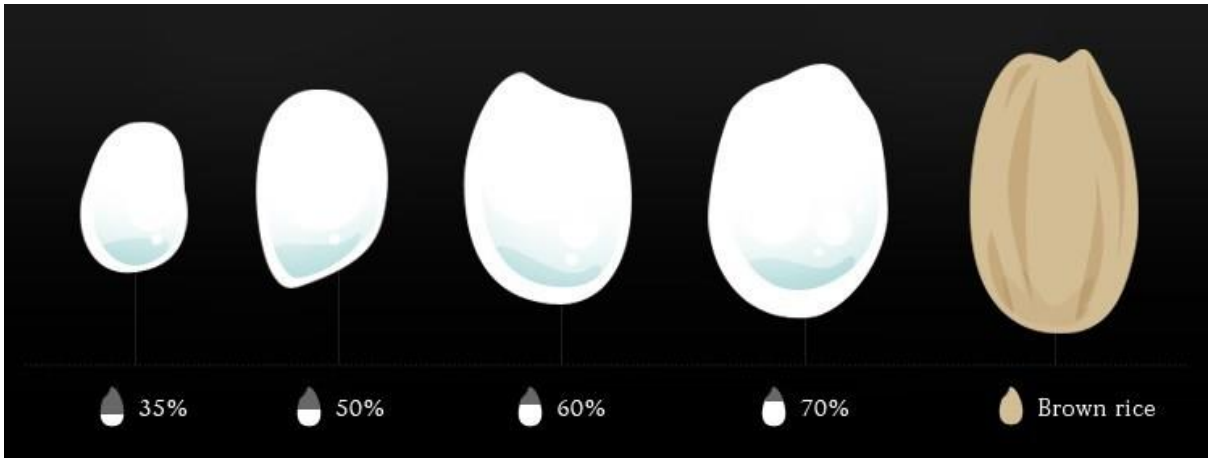

Figure 2.3 Polishing ratio (*Seimai-buai*) of rice

2.6 Water

Water is an important material used in *sake* brewing, accounting for about 80% (v/v) of *sake*. The water for *sake* brewing must be colorless, tasteless and odorless; it must also be neutral or weakly alkaline, containing only traces of iron, ammonia, nitrate, organic substances, and micro-organisms. In particularly, iron ions are injurious to *sake*, giving it a color and engendering deterioration. Therefore, iron in brewing water is removed using appropriate treatments such as aeration, successive filtration, adsorption (with activated carbon or ion-exchange resins) and flocculation.

Sake in its completed form is about 80% pure water. There are a number of elements whose presence is indispensable, without which certain steps of the brewing process will not proceed smoothly. There are also several things that are only detrimental, and either impede the process or adversely affect the *sake* in other ways. Magnesium, calcium, phosphates, and potassium are great for sake, and are valued because they help support yeast and koji. On the other hand, some minerals such as iron and manganese have adverse effects on *sake* quality.

Iron will darken the color of *sake* and adversely affect its taste and fragrance. This happens because it chemically attaches to the center of a normally colorless compound attached to an amino acid produced while the *koji* rice is being made. Also, as *sake* ages, the residual sugars react with amino acids present to change the flavor and smell, and the presence of iron hastens this reaction. Manganese plays a different but equally despicable role. When sake is exposed to light, in particular ultraviolet light, manganese promotes a chemical reaction that will discolor and de-luster the appearance of a *sake*.

Potassium, magnesium and phosphoric acid are necessary for the propagation of the yeast in the “shubo” (yeast starter), as well as in the proper development of good *koji*. If these are not present in sufficient amounts the yeast cells will not multiply as well or as

quickly, throwing off the timing off of the entire fermentation so that it can be properly controlled (John, 2016).

2.7 Washing, soaking and steaming of rice

After polishing the grains have rice dust residue all over them, left over from the polishing process. This dust is made up of the outer and middle layers that the brewer wanted to remove and therefore it needs to be washed off. Soaking and then steaming is necessary to soften the grain and ensure that it has the correct texture and level of moisture so that it can break up into the water during fermentation.

2.8 Koji rice making

Koji rice is cooked rice that has been inoculated with *Aspergillus oryzae*. The mold releases enzymes that ferments the rice by decomposing its carbohydrates and proteins. Enzymes of about 50 kinds have been found in *koji*, the most important of which are amylases. α-Amylase and saccharifying amylase (Exo-α-glucosidase) play important roles in amylolytic action. Furthermore, proteases of some kinds are also important enzymes: acid-proteases and alkaline-proteases are found in koji. In *sake* mash, the enzymes decompose protein to form amino acids and peptides (oligoamino acid) at low pH values such as pH 3–4. Furthermore, amino acids or peptide-supported yeast grow with food or nutrition. The enzyme acts indirectly, decomposing rice protein while combining to an active site of the α-amylase (William & Aoyagi, 2012).

2.9 Yeast and seed mash

Fermentative multi-budding yeast, Saccharomyces cerevisiae, which has been used not only in *sake* brewery, but also in beer brewry, winery and baker. 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 (Walker & Stewart, 2016). However, the yeast was distinguished from other strains of S. cerevisiae by additional properties such as vitamin requirements, acid tolerance, sugar osmophilic character, and adaptability to anaerobic conditions. Additionally, *sake* yeast has advantageous features that enable its growth under high sugar contents and low pH conditions, to produce *sake* under open system fermentation. *Sake* yeast formed a large amount of foam during main mash fermentation. Because one third of the capacity of the fermentation vessel is occupied by foam during usual main fermentation, preventing foam formation would be greatly advantageous to breweries to save space occupied by the foam and scaling up the amount of *moromi* produced. Some largemolecular-weight compounds that arise from steamed rice grains are also regarded as taking part in foam formation.

Recently, foam formation has involved existing proteins, with foam formation on the yeast surface (Harandi *et al*., 2017).

2.10 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.

In *sake* brewing, *moto* is important as a yeast starter for the fermentation of *moromi*. *Moto* is necessary to provide a pure and abundant yeast crop, and to supply sufficient lactic acid to prevent contamination of harmful wild yeast or bacteria during *moto* production or in the early stages of main fermentation. In *sake* brewing, temperature control is also extremely important to balance saccharification and fermentation, both of which occur simultaneously in *moromi*. Therefore, we call it 'Parallel Fermentation'. The fermentation temperature is usually in the range of 8-18˚C. Small quantities of sugars released from steamed rice and *koji*are fermented gradually by *sake* yeast until the alcohol content reaches nearly 20% (v/v) (Keller dalam Dwiyanti, 2008).

2.11 Mash filtration

After alcohol fermentation, the mash is divided into *sake* and solids by filtration. The mash is poured into bags made of synthetic fiber, which are laid in a rectangular box. *Sake* is squeezed out under hydraulic pressure. After complete filtration, the solids pressed in a sheet are stripped out of the bags. Recently several automatic filter presses for filtering *moromi* mash have been used.

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. Sakekasu is highly nutritious and can be eaten as is or used as a raw ingredient for making shochu-traditional Japanese distilled liquor.

2.12 Sedimentation and filtration

The slightly turbid *sake* is clarified to separate lees by standing in a vessel for 5–10 days at a low temperature. 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 (Nrib, 2013).

2.13 Pasteurization

After settling the clarified *sake* for a further 30–40 days, *sake* is pasteurized, killing yeasts, harmful lactic acid bacteria, and enzymes. *Sake* is heated to 60–65°C, passing it through a helical tube type heat exchanger for a short time. Recently plate-type heat exchangers with high efficiency of heat transfer have become available**.**

2.14 Aging (maturation)

Usually *sake* is aged and stored for a short time. It does not age for a long time of several years or longer. Vintage wine is aged much longer than *sake.* During storage, *sake* matures gradually. The maturation process is probably the result of oxidation reactions and physicochemical changes. *Sake* changes and adopts a smoother taste. The storage temperature should be maintained carefully at 13–18°C, with consideration being devoted to the rate of maturation and the time of bottling.

Furthermore, the *sake* taste is smooth and less stimulated by ethanol because of molecules of ethanol and water flocculate in the *sake* during aging. However, research of *sake* aging has been conducted by many researchers.

2.15 Adjustment and packaging

Water is nearly always added to sake before it is bottled. This reduces the alcohol level from around 20% to between 15% and 17%. Nearly all sakes are packaged in glass bottles of various sizes.

2.16 Types

According to Japan *sake* and *sochu* maker association, *sake* is classified as follow:

2.16.1 *Junmai*

This can be translated as pure rice *sake*. Nothing is used in its production except rice, water, and *koji*, the magical mold that converts the starch in the rice into fermentable and non-fermentable sugars. *Junmai* is made with rice that has been polished (milled) so that at least 30% of the outer portion of each rice grain has been ground away. The taste of *junmai* is usually a bit heavier and fuller than other types, and the acidity is often a touch higher as well.

2.16.2 *Honjozo*

Honjozo is sake to which a very small amount of distilled ethyl alcohol (called brewers alcohol) has been added to the fermenting sake at the final stages of production. (Water is added later, so that the overall alcohol content does not change.) *Honjozo*, like *Junmai*, is made with rice that has been polished (milled) so that at least 30% of the outer portion of each rice grain has been ground away. This, plus the addition of distilled alcohol, makes the sake lighter, sometimes a bit drier, and in the opinion of many, easier to drink. It also makes the fragrance of the sake more prominent. *Honjozo* often makes a good candidate for warm sake. Note that most run-of-the-mill cheap sake has an excessive amount of brewers alcohol added to it, which is not good. *Honjozo* has only a very small amount of added alcohol.

2.16.3 *Ginjo*

This is sake made with rice that has been polished (milled) so that no more than 60% of its original size remains. In other words, at least the outer 40% has been ground away. This removes things like fats and proteins and other things that impede fermentation and cause off-flavors. But that is only the beginning: *ginjo* is made in a very labor intensive way, fermented at colder temperatures for a longer period of time. The flavor is more complex and delicate, and both the flavor and the fragrance are often (but not always) fruity and flowery. Click here for flavor profile and further details on *ginjo*.

2.16.4 Daiginjo

Daiginjo is *ginjo* made with rice polished even more, so that no more than 50% of the original size of the grain remains. Some *daiginjo* is made with rice polished to as far as 35%, so that 65% is ground away before brewing. *Daiginjo* is made in even more painstaking ways, with even more labor intensive steps.

2.16.5 *Namazake*

Namazake is *sake* that has not been pasteurized. It should be stored cold, or the flavor and clarity could suffer. *Namazake* has a fresh, lively touch to the flavor. All types of sake (*junmai, honjozo, ginjo and daiginjo*) can be *namazake*, or not.

2.17 Factors influencing types and varieties of sake

According to Japan sake and *sochu* maker association, the factors influencing types and varieties of sake are presented in figure below.

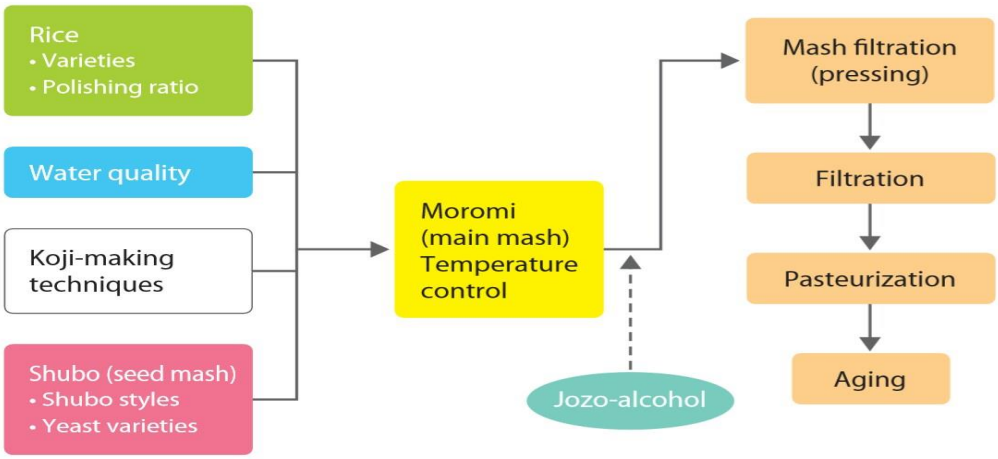

Figure 2.4 Factors influencing types and varieties of sake (*Sake* Association, 2011)

2.18 Rice varieties

The best types of rice for brewing sake contain less protein. The grain should be firm and not easily break during the rice polishing process. Sake rice typically has a bigger grain than table rice and has a lot of starch at the center of the grain called “shinpaku”. Because shinpaku is white and very soft, it easily absorbs the *koji* mold which converts the starch into sugar. The individual character of a particular sake is due in part to the variety of sake rice used in brewing. Currently there are approximately 100 kinds of sake rice grown in Japan. Most of *sake* breweries use Calrose or Yamada *Nishiki* (sakebrewclub 2018).

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

The polishing ratio (*seimai-buai*) is an important factor in sake brewing; a high polishing ratio (more of the grain remains after polishing) yields complex, rich *sake* that have a stronger rice flavor. Conversely, a low polishing ratio produces lighter *sake* with a cleaner taste. The core of the rice grain is rich in starch, while fats, proteins and amino acids are concentrated near the surface. The fat and protein is nutritious for eating, so the rice-polishing rate for table rice is around 90%. On the other hand, fat and protein have strong flavor profiles that, in excess, can adversely affect the flavors and aromas of *sake*, resulting in an unpleasant product. This is why rice-polishing is a vital step for making *sake*.

Sake made with unpolished rice has a comparatively deeper, thicker taste, giving the impression of a strongly flavored *sake* with a noticeable smell of rice. But when sake is made using well-polished rice, the aroma is bright, with a flavor that leaves a light and clear impression.The rice-polishing ratio or mill rate shows how much of the rice surface is removed. For instance, when the ratio is 70% that means 30% of the outer surface is

polished away. In general, the more the rice is polished, the higher the grade of *sake*. Highly polished rice usually leads to a lighter, more complex flavor profile (Wylie, 2019).

2.21 *Koji* making

Wine is fermented from grapes, which already contain sugar (glucose, to be chemically correct). This is what yeast cells need for food. There are other kinds of sugars, but they cannot be metabolized by yeast. So in winemaking, yeast is added to a liquid already containing sugar. Beer and other beverages made from malted barley begin not with sugars, but with starches, which are molecularly monstrous. Here, brewers employ enzymes brought out in the barley malting process (where the barley is moistened and warmed, i.e. the sprouting process begun, albeit artificially) to break down the starches into sugars.

Sake is brewed from white rice stripped of its husk. There can be no malting, so the starch-chopping enzymes must come from somewhere else. Enter the cooperative *koji*. The dark-green spores, sprinkled onto steamed rice, graciously provide the necessary enzymes for saccharification. There are many enzymes involved in this process. Some act to create fermentable sugar (glucose), others act more to create sugars that will not ferment but will instead affect texture and flavor in a sake.

Koji mold produce 50 different kinds of enzymes, the most important are amylases and proteases. The amylase decomposes and liquefies starch, and glucoamylase forms glucose and thus regulates yeast growth and fermentation. The acidic protease decomposes proteins to form peptides and helps amylase action. Cultural conditions influence the production of enzymes by the molds. In general, the higher the culture temperature (up to 42 ^{0}C), the greater the amylase activity. Lower temperature favors development of protease activity. As culture times become longer, especially at the late stage of *koji* preparation, more enzymatic activities appear in the *koji* (Okuda *et al*., 2019).

2.22 Types of yeasts

Converting glucose to alcohol, yeasts also produce by-products such as acid, amino acid, aroma components (ester) and the like which all have substantial effects to the aroma of sake. The nature of those by-products are different depending on the types of yeasts. Also the temperature at which they are most active varies with the types of yeasts.

Nowadays, due to the unpredictable and wild nature of ambient strains, many breweries use cultures, the majority of which they purchase from the Brewing Society of Japan. The yeast that the society distributes are called kyokai-kobo (literally, society yeasts). There are currently 20 varieties, each identified by a number from 1-20. No.1 through 5,

the first varieties to be distributed back in the early 1900s, and No.8 are no longer distributed. Single digit kyokai-kobo**,** such as No.6 and No.7, don't boast much in the aroma department but are reliable strong fermenters. Double digit kyokai-kobo, such as 14 and 16, have won a section of the brewing community over with their elegant floral and fruity aromas, an aroma that is called a *ginjo* aroma.

2.23 *Shubo* (seed mash) production process

Seed mash production process is classified into two types according to the process by which it is acidified as seed mash acidified by naturally occurring lactic acid bacteria, and seed mash with added lactic acid. The former type is called Kimoto style, which has been produced traditionally; the starter culture was a great technique. In contrast, in the latter type, sake yeast in pure culture is inoculated at the first step in the seed-mash process. Sugar accumulates to a high content (over 20%) at an early stage in both seed-mash processes. This, together with the acidic condition, is considered to prevent contamination by other microorganisms and to facilitate the predominant growth of sake yeast.

The procedure for making the lactic-acid-added seed mash varies depending on the brewers. Some breweries use a large amount of compressed sake yeast cells harvested from an aerobic propagation culture in place of the seed mash (Okuda *et al.*, 2019).

2.24 Use of alcohol and other ingredients

Very small amount of distilled ethyl alcohol (called brewers alcohol) has been added to the fermenting sake at the final stages of production. (Water is added later, so that the overall alcohol content does not change.) Honjozo is sake wherein a small amount of distilled pure alcohol is added to smoothen and lighten the flavor, and to make the sake a bit more fragrant. Honjozo-shu, like Junmai-shu, must be made with rice with a Seimai Buai (degree of milling) of at least 70%. In addition to 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 alcohol or ingredients have been used.

2.25 Mash filtration (pressing), secondary filtration

Once the fermentation has finished, all sake must be filtered through synthetic fiber bag. This removes the rice solids and produce slightly cloudy liquid. It is then filter to produce a clear liquid. Slightly cloudy sake is pass through the bag is called arabashir (the first run). The cloth bags are left to drip out slowly on their own is called nakatori. Finally the bags are press out to last of sake, this sake is called oshikiri (clear sake).

2.26 Pasteurization

In traditional sake process, there are still few yeasts and enzyme in sake, and may contaminant by Bacteria at the storage or bottling stage. That's why most sake will pasteurize twice before storage stage or bottling stage. The pasteurize mothed in *sake* process called Hiire. The Hirre method is keeping the temperature in 62℃ to 68℃ and making yeast, enzyme, and bacteria denature. Alcohol and flavor compounds will escape with the heating stage, that why sake after Hiire method will lose some delicate flavor.

2.27 Aging of *sake*

While *sake* is traditionally thought of as not being suitable for aging, and best consumed within a year. After aging, the aroma and color of sake change. Aroma turns milder, and some smell like coffee or roasted caramel. Color turns to be golden like an amber. In general, less polished sake categories like Junmai and Honjozo, aged in a relatively high temperate around 15°C would become a *sake* of richly aged type. More polished sake categories like *Ginjo* categories, aged in a relatively low temperature around 5°C would become a sake of slightly aged type. The taste of *sake* after aging becomes round and rich. Both sake meter value and acidity increase. Although aged sake tastes great and unique, it is not so widely known yet. Even in Japan, not many Japanese have tried aged *sake*. That is also why aged *sake* is still ridiculously cheap now. But when people realize its goodness in the future, it must become more and more expensive and difficult to get, like some aged whisky.

2.28 Use of starter culture for fermentation in Nepal

2.28.1 *Murcha*

Murcha is a starter culture used for the production of alcoholic beverage by different ethnic group in Nepal, Bhutan and India. It is a white coloured cake made up of rice flour and different kinds of wild herbs. In eastern Nepal, the *murcha* producer uses more than 42 plants (two ferns, five monocots, and 35 dicots) and their roots and leaves for making *marcha* (Tamang 2016). In Nepal, *murcha* is prepared mostly by Rai, Limbu, Tamang, Gurung, Newar and Tharu communities. *Murcha* cakes are mildly acidic (pH 5.2) and contain 13% w/w moisture and 0.7% w/w ash (dry weight basis) (Tamang & Sarkar, 1995).

Microbiologically *murcha* is a mixed culture and consists of saccharifying molds, fermentative yeast, and acidifying lactic acid bacteria. The microbiology of the Nepali murcha starters was analyzed for first time in the early 1990s. Most of the isolates belonged to Pediococcus pentosaceus. The identified yeasts were Saccharomycopsis fibuligera, Pichia anomala, and Saccharomyces spp. The molds were Rhizopus and

Mucor, all members of the Mucorales (Dahal *et al*., 2005). The murcha starter cakes are of two types, manapu and Mana. The manapu is prepared from rice flour and millet grains, whereas the Mana is prepared from wheat flakes (Shrestha *et al.,* 2002).

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 substance (Tamang & Sarkar, 1995).

2.28.2 Yeast

The yeast species that dominates in the production of alcoholic beverages worldwide is Saccharomyces cerevisiae, and the particular strains of this species employed in fermentation exert a profound influence on the flavour and aroma characteristics of different beverages. For large-scale beverage fermentations, as in brewing, winemaking and distilled spirit production, pure cultures of selected strains of S. cerevisiae are usually used (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. S. fibuligera, which was the only starch-degrading yeast found in murcha (Tamang & Sarkar, 1995).

2.28.3 Mold

Mold is a type of fungus that consists of small organisms found almost everywhere. They can be black, white, orange, green, or purple. They also play important roles in biotechnology and food science in the production of various foods, beverages, antibiotics, pharmaceuticals and enzymes. The majority of molds involved in these fermentations belong to the genera Aspergillus, Penicillium, Rhizopus, Amylomyces and Mucor. Species of Neurospora, Monascus and Actinomucor are also involved in some fermentations. Species of Aspergillus and Penicillium are typical of products from temperate areas (cheese, meat, soy sauce), whereas Rhizopus, Amylomyces and Mucor are typical of products in predominantly tropical regions (tempe, tape) (Chávez *et al.*, 2011).

There are different kinds of molds present in marcha such as Mucor circinelloides, Rhizopus chinensi and Amylomyce. Amylomycemolds produce enzymes like α-amylases

and β-amylase. α-amylase breaks down long chain carbohydrates, ultimately yielding maltotriose and maltose from amylose, or maltose, glucose and "limit dextrin" from amylopectin. 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. 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 (Pervez *et al*., 2014).

2.29 Some important traditional alcoholic beverages of Nepal

Among the various fermented foods, Jaand,Chhyang, Tongba,Poko and Rakshi are the major alcoholic fermented liquor traditionally consumed in various parts of Nepal, depending on the availability of the raw materials.

2.29.1 *Jand*

Jand is an undistilled alcoholic beverage made from a wide range of carbohydrate-containing substrates. The basic raw materials for the production of *jand* are rice, millet, maize, wheat, or other substrates containing starch. *Jand* refers to the sweet-sour cereal beer made from grains like finger millet, rice, wheat (Triticumspp.), and maize (Zeamays) by using murcha.*Jand* finds a very prominent place in ethnic groups of the Nepalese that includes Magar, Gurung, Rai, Limbu, Sherpa, Bhote, and Lepcha castes it is also used in several festive occasions, rituals, and rites, settling disputes, and appeasing deities (Dahal *et al*., 2005).

The fermented mash, *jand*, is consumed in different ways. It is squeezed with added water, strained in a traditional bamboo-made chhapani or aluminum strainer, and the whitish cloudy extract thus obtained is served in deep bowls, tumblers or other containers. Alternatively, the fermented mash is put into a wooden or aluminum cylindrical vessels, hot water added over it and the extract sucked with a help of bamboo or aluminum tube.

The first step in jaand fermentationis the saccharification and liquefaction of the starch, and the second is the utilization of simple sugars to produce alcohol and CO_2. The role of fungi presents in marcha to produce amylase needed to saccharify and liquefy starch. The amylase activity has been reported to reaches its peak on the second day of fermentation. The presence of mixture of yeasts (Pichia anomala, Saccharomyces cerevisiae, Candida galbrata) and lactic acid bacteria (Pediococcus pentosaceus, Lactobacillus bifermentans) utilize simple sugar and produce alcohol and CO_2 (Rai, 2018).

A brief outline of jaand preparation of bhatte jand (from rice) is shown in Figure below (Tamang *et al.,* 1996). The sensory quality of jaand is naturally dependent on its physicochemical properties, which in turn are dependent on several other factors, including the quality of *murcha.*

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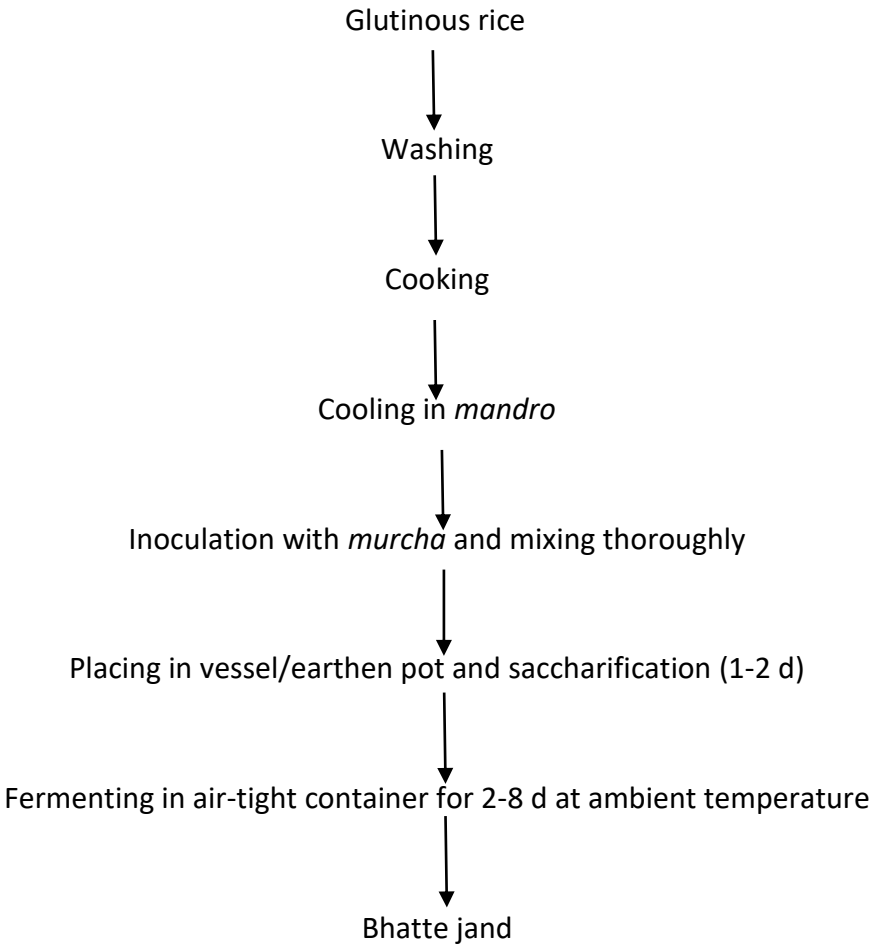

Figure 2.5 *Bhatte jand* preparation

2.29.2 Chhyang/ Thon

Thon, pronounced thon (with a very strong 'n' sound at the end) in Newari and Chhyang in the Sherpa language, it's a milky, refreshingly sweet alcoholic drink made by fermentation of rice. This is a special drink used by Newar communities during festivals, social events, and family celebrations. Jyapus (Newari farmers) prepare it during the rice planting and harvesting time. It is little cloudy in color and has a mild sour taste, much like a mild cider. It is probably the second most consumed local drink in Nepal.

Traditionally, *Chhyang* was served in brass bowls and some places still do. Today, they are typically served either in normal glasses, steel bowls or even plastic bottles. Most commonly available type is 'white *Chhyang*', the kind that appears milky. It can be brewed in two weeks. Lesser known are *'Karthon'* a thick brown *Chhyang*, and

'Hyaunthon' red *Chyang*. Both tastes similar to cider, but *Karthon* is not as widely preferred as its white cousin. It does pack a good punch though. *Hyaunthon* is also mild and would be preferred if not for the price that is nearly ten times that of the white *Chhyang* (Ray *et al*., 2016).

2.29.3 *Tongba*

Commonly pronounced as tum-baa by locals this is the most popular alcoholic beverage in cooler parts of eastern Nepal. Like *Chhyang*, it is popular among Rai's, Limbu's and Sherpas, but mostly in eastern regions of Nepal. It is also highly popular further east in Sikkim and Darjeeling, India. Unlike most alcoholic beverages, millet is brewed without additional water. Technically it falls in the category of beer but unlike beer. Boiled millet is fermented for a week or two. For consumption, container-full brewed millet is served and then hot water is added. Alcohol in fermented millet is soaked by the hot water that is drunk through a wooden or metal straw. Traditionally they are served in a large wooden container called *Tongba*, hence the name.

2.29.4 *Poko*

Poko is ethnic, mild alcoholic beverage prepared from rice in Nepal using manapu. It is creamy color, soft texture, juicy, sweet and sour taste with mildly alcoholic and aromatic flavor. This product is widely used by the rural people in central Nepal especially in Kathmandu during occasions like weddings, festivals, cultural celebrations as well as special offerings to goddesses. People believe that poko promotes good health, nourishes the body and provide vigor and stamina (Shrestha & Rati, 2003).

Traditional method of *poko* preparation is shown in figure below.

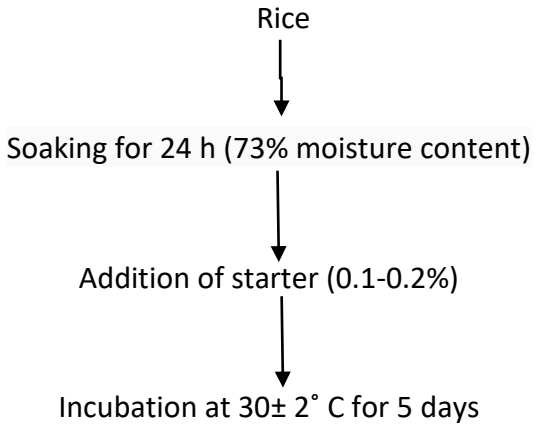

Figure 2.6 A protocol for *poko* prepration

2.29.5 *Rakshi*

Rakshi is distillate major traditional alcoholic products of Nepal. It is the common drinks traditionally prepared by different ethnic group in Nepal. *Rakshi* is an unaged congeneric

spirit obtained by pot distillation of the slurry of *jaand. Rakshi* is like whiskey and has highly varying alcohol content. Traditionally rakshi is made by using marcha as starter culture as that in preparation of *jand* (Tamang, 2016).

After fermentation, the mash is steam distilled in atraditional distillation apparatus. The apparatus generally contains three sections—bottom, center, and top. The fermenting mash is placed in the bottom section, and the apparatus is set over a fire. In the middle section, there is a distillate-collecting bucket, and at the top, the cold water pot for condensing alcohol vapor. Generally, the cold water pot is replaced by new cold water and accordingly *rakshi* is named, viz, one time water change- ekpane, two tomes-duipane etc. First distillate contains higher high in alcohol content up to 80-90% (Dahal *et al.*, 2005).

2.30 Physico-chemical and microbial changes during fermentation and aging

2.30.1 Esters

Esters are the class of volatile compounds that are responsible for “fruity” smell in wines. They are some of the most abundant aromatic compounds in wine. Esters are found in fruit juice in small amounts, but most of the esters in wine are formed during fermentation or during wine ageing (Lambrechts & Pretorius, 2019). Esters can be classified as either volatile esters (or neutral esters) and acid esters (or non-volatile esters). The neutral esters are produced through enzymatic reactions; acid esters are formed in simple hydrogen-ion-catalysed esterification (Margalit, 2004). This simple acid catalysed reaction is slower than enzymatic esterification, but may be responsible for aged characters of wine. Additionally, acid catalysed esterification may occur faster in wines of a lower pH. Therefore, esters not only contribute significantly to the sensory impact of newly fermented wine, but the aged product as well.

Volatile esters are produced in such high quantities during fermentation that the concentration surpasses the synthesis/hydrolysis equilibrium point, and they cannot be maintained. During ageing, volatile esters decrease as they react hydrolytically and finally achieve equilibrium. Non-volatile esters contribute relatively negligible aromas and flavour in wine. Native yeasts such as Hansenulaanomala and Kloeckeraapiculate produce an abundance of ethyl acetate. Therefore, yeast strain can affect the formation of certain esters. Lema *et al*. (1996) found that the concentration of total esters was more dependent on the size of the initial yeast culture, rather than the yeast strain itself. However, the concentration of the esters produced was different from strain to strain. Saccharomyces yeast generally produce roughly the same concentrations of esters, but their distribution differs. Non-Saccharomyces yeast can produce many more

esters than Saccharomyces, but may not always be pleasant. Nonetheless, this may be a reason why natural fermentations produce wines of greater complexity (Boulton *et al.,* 1996).

2.30.2 Aldehyde

Aldehyde are synthesized by yeast as intermediate in the fermentation of alcohol through the decarboxylation of keto acids. Acetaldehyde constitutes around 90% of all the aldehydes found in wine (Lambrechts & Pretorius, 2019). During alcoholic fermentation sugars are converted to ethanol and carbon dioxide, but the process is not so simple and other by-products like acetaldehyde are also formed during the process. Acetaldehyde is, however, not only formed during alcoholic fermentation, but also as an intermediate product during the bacteriological conversion of ethanol to acetic acid by acetic acid bacteria. At low alcohol concentrations and high oxygen exposure, significant concentrations of acetic acid is formed, but at the higher alcohol concentrations of wines and limited oxygen exposure, acetic acid bacteria tend to produce more acetaldehyde. Although yeasts usually reduce acetaldehyde to ethanol, it can oxidise ethanol to acetaldehyde in oxidative circumstances, during more oxygen exposure when containers are not kept full. With certain winemaking methods, like in the case of sherry, such oxidative circumstances are promoted to favour this process. During the natural maturation of wine acetaldehyde can also be formed. This process is, however, rather of chemical than microbiological nature (Osborne *et al*., 2000).

Acetaldehyde is usually associated with its negative impact on wine quality. Although it is mainly formed during alcoholic fermentation it can also occur in wine through other ways. At concentrations lower than 70 mg/L it can impart a fruity flavour to wine, which often occur in freshly fermented wine. At higher concentrations from 100 to 120 mg/L it can, be produce pungent smell in wine (Swiegers *et al*., 2005).

2.30.3 Organic acids

A great variety of organic acids present in alcoholic beverages play significant roles, as they affect the organoleptic properties of the product (for example, taste, aroma and colour), stability, nutrition, acceptability and quality. Moreover, the level of organic acids in beverages such as beer, juice, wine and others, contributes much to tartness, other flavour attributes and longer product shelf-life(Shale et al., 2013). During alcoholic fermentation several important organic acids, such as succinic, pyruvic, lactic and acetic acid, are produced by yeast and bacteria and are mainly associated with the fresh, tart, sour and sometimes metallic taste of wines (Chidi *et al.,* 2018).

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).

2.30.4 Higher alcohols (fuel oils)

Alcohols with more than two carbon atoms are commonly known as higher alcohol or fusel oils, higher alcohols occur naturally in alcoholic beverage as by-product of alcoholic fermentation. Higher alcohols are considered as one of the important flavouring compounds in alcoholic beverages. The major higher alcohols present in alcoholic beverage are 1-propanol, 2-butanol, iso-butanol and isoamyl alcohol(Lachenmeier *et al.*, 2008).

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 2methylpropanol (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).

2.30.5 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^{o}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 with1.8×10^6 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 pH, total acidity, alcohol and reducing sugar contents during the traditional fermentation of Bhatti jand and found that the pH decreased from 6.1 (day 0) to 3.96 (day 10) during fermentation at 28˚C. A large drop in pH was recorded in the first day of fermentation (from 6.1 to3.36); there was no drastic change in pH 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.

In traditional sake yeast (Saccharomyse sake) is added at the rate of 105 to 106 cfu/g and eventully propagates to level of 3x108 to 4x108 cfu/g (Murakami, 1972). During takju preparation (a Korean traditional wheat based alcoholic beverage) molds disappeared after 2 or 3 days of fermentation (Sharma *et al.*, 2020).

CHAPTER 3

MATERIALS AND METHODS

Traditional starter culture, *murcha*, were collected from different district of Nepal. Those *murcha* sample were prepared by using local raw material by traditional method and paddy collected from local market of Saptari.

3.1 The starter culture (murcha) collection

Starter culture were collected from different places of Nepal. The list of collected *murcha* sample with their labelling in this research is given in Table 3.1.

Table 3.1: List of *murcha* sample collected from different places of Nepal

S.N	Place of murcha collected	Murcha labelling
1	Rajbiraj	Rm
2	Siraha	Sm
3	Dharan	Dm
4	Tamghas	Tm
5	Phidim	Pm
6	Lalitpur	Lm
7	Bhaktapur	Bm

3.2 Morphological analysis of molds and yeast

All isolates were cultured on the PDA (potato dextrose agar) plates, their morphological characteristics were observed based on colour, texture, margin and elevation. For this, the yeast were stained with cotton blue and was observed under microscope. However, for molds cellotape method (Chim *et al*., 2015) was used. For this, small piece of clear cellotape was used to stack the mycelial fragments. Mycelial fragments along with some spore stacked to the tape were observed under microscope by staining with a drop of methylene blue.

3.3 Starch hydrolysis test

The molds isolates were inoculated on PBSA plates containing 1% starch and were incubated at 28^{o}C for 3-4 days. Then, the plates were flooded with iodine solution and were observed for clear transparent zone around the colony.

3.4 Preparation of crude enzyme solution

Isolated molds were cultivated in 100 ml Czapek's broth medium which consists of: sucrose 20 g/L, $NaNO_3$ 2 g/L), KH_2PO_4 1 g/L, KCl 0.5 g/L, $MgSO_4.7H_2O$ 0.5 g/L, $FeSO_4.7H_2O$ 0.01 g/L and $CuSO_4.5H_2O$ 0.05 g/L, carboxylmethyl cellulose 5 g/L and at pH of 5.6. The medium was inoculated with $1*10^6$ cells and allowed to grow at 28 ^oC on rotary shaker at 100 rpm for 7 days. Fungal mass was removed by filtration and the filtrate was centrifuged at 10,000 rpm for 10 min at 4 ^oC using refrigerated ultracentrifuge. Then, the produced crude enzyme was added with few drops of toluene and stored at 4 ^oC (Begum & Alimon, 2011).

3.5 Calculation of saccahrification percentage

To determine the percentage of saccharification, 100 mg dry weight equivalent of polished rice were mixed with 1 ml of 0.1 M citrate buffer (pH 5.5) and incubated with 1 ml crude enzyme at 50 ^oC for 48, 72, 96 hrs. The amount of reducing sugar released was measured by the DNS method. Saccharification was calculated by applying the equation of Spano et al. (1976).

Degree of saccharification(%)= (reducing sugar × 0.9 × 100)/ substrate

Where 0.9 is used for glucan to glucose conversion.

3.6 Liquification test

Inoculum of best mold from starch hydrolysis test was prepared in PBSA broth. Then, the prepared inoculums was inoculated into a conical flask containing steamed rice. The flask was sealed with cotton plug and incubated at 28 ^oC for 3 days.

3.7 Based on colony fluorescence

The isolate that showed positive starch hydrolysis test and positive liquification test having higher saccharifying capacity was grown in CMA (coconut agar media) plate for 3 days. Then, the plate was exposed to UV radiation at 365 nm. Obeservation was done for formation of fluorescence ring around the colony of mold.

3.8 Aammonium hydroxide vapour-induced colour change test

Aflatoxin producing and non-producing strain was identified by the method of Satio and Machid (1999). In this method a single colony of the isolate that showed positive starch hydrolysis test and positive liquification test was grown on YES (Yeast Extract Sucrose) plate. Then, the plate was inverted and 2 or 3 drops of concentrated ammonium hydroxide solution was dropped onto the inner side of the plate lid. Observation was done for plum-red colouration. Aflatoxin positive isolate produces plum red colouration.

3.9 Inoculum preparation

The inoculums of yeast and mold were prepared in YEPD broth and PBSA broth respectively. The yest was used for fermentation while mold was used for koji preparation

3.10 Koji preparation

Koji is one of the most important constituents during the preparation of rice wine. During koji preparation (see Figure 3.1) first paddy was milled and bran was removed. The degree of polishing of rice for koji preparation was similar to common rice used in our kitchen. Polished rice was soaked in water for about 24 h followed by steaming for 1 h. After steaming, rice was placed in tray and mold inoculum (5% v/v) was inoculated. Then, the set up was left for 2 days at room temperature. After 2 days, the whole mass was dried at 70^{o}C for 5 h.

3.11 Fermentation process

For fermentation standard ratios of steamed rice, koji and water placed in the fermentation tank were steamed rice 35%, koji 8% (expressed as ratios of polished rice) and water 57%. 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 ^{o}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 table below.

3.12 Racking and filtration

When the fermentation was complete (see Figure 3.2), 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.

3.13 Charcoal clarification

Finally, active charcoal was used for decolouring, flavour adjustment and control of the aging process.

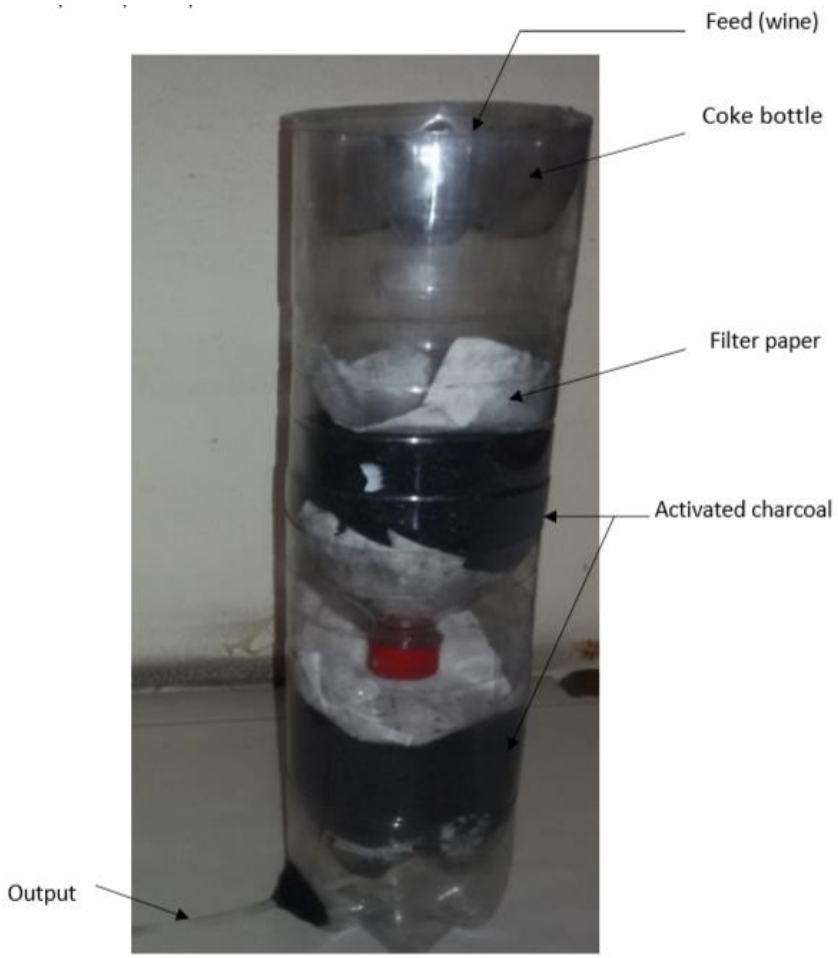

Figure 3.3 General setup prepared for charcoal clarification

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 Figure 3.3. Yellow turbid rice wine was passed through first and second bottle and finally collected in third bottle (see Figure 3.4). After filtration sake was kept in maturation for 6 months(see Figure 3.5).

3.14 Chemical analysis of rice wine

During fermentation process different parameters were measured at 2 days interval. The pH was measured using pH meter (Corion Research, USA).Total soluble solid (TSS) was measured by ERMA hand refractometer having a range of 0-32°Brix at 20 °C.

Similarly total titrabel acidy was measured by the method of sochu and sake brewing association. For this 10 mL of the fermenting wine sample was taken in 100 mL volumetric flask and volume was made upto 100 mL. From this 10 mL of diluted sample 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 acid / L.

After complete fermentation, rice wine thus produced was analysed for ethanol content, methanol content, volatile acidity and ester concentration.

3.14.1 Measure of ethanol content

About 250 ml of sake was taken in a round bottom flask. Then, it was boiled at distillation setup until the volume reduced to the half of the orginal volume. The distillate was collected in a beaker and the volume of it was adjusted to 250 mL by adding double distilled water. Then, specific gravity was measured by using specific gravity bottle and refractive index (given in Brix equivalents) was measured with a refractometer. Alcohol by volume of *sake* was calculated by using the formulae given below.

Ethanol (% Abv) = 1.646 × RI – 2.703 ×(145 – 145 / SG) – 1.794

Where,

S.G. (specific gravity) = (distillate mass / volume) / (mass of water / volume); and RI is refractive Index of distillate given in Brix equivalent

3.14.2 Measure of methanol content

Methanol of *sake* was determined by spectrophotometric process in this process 50 mL of sample was taken and distilled, collected about 40 mL of distillate. Dilute 1 mL of distillate to 5 mL with distilled water and shaken well. And then 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. Added 2 mL of $KMnO_4$ reagent to each test tube keep aside for 30 min.

Decolourized he solution by adding a little sodium bisulphite and add 1 mL of chromotropic acid solution. Mixed well and added 15 mL of sulphuric acid slowly with swirling and place in hot water bath maintaining 80 ˚C for 20 min. Observed the colour development from violet to red. Cooled the mixture and measure the absorbance at 575 nm using 1 cm cuvette cell.

Calculations:

$$\text{Methanol}\left(\%\frac{\text{V}}{\text{V}}\right) = \frac{\text{sample OD}}{\text{standard OD}} \times 0.025 \times \text{dilution factor}$$

3.14.3 Determination of volatile acidity

Volatile acidity as acetic acid was determined using the methods described by Amerine and Ough 1980. In brief 50 ml of distillate was titrated against standard 0.05 N NaOH using phenolphthalein as an indicator. Where 0.05 N NaOH is equivalent to 0.003 g of acetic acid.

3.14.4 Determination of ester

Ester as ethyl acetate was determined using the methods described by Amerine and Ough 1980. In brief, 10 ml of 0.1 N NaOH was added to the distillate to neutralized the sample obtained from the volatile acidity determination, and so obtained solution was refluxed on refluxing apparatus using heating mantle for one hour. Cooled and back titrated the unspent alkali against 0.1 N H2SO4. Taking 50 ml of distilled water instead of distilled blank value was determined. Simultaneously in same way, the difference in the titer value in ml of standard 0.1 N H2SO4 gives the equivalent ester.

Note: 1 ml of 0.1 N H2SO4 is equivalent to 0.008 g of ethyl acetate.

3.14.5 Determination of essential oils by GC/MS

When GC is combined with MS, a powerful analytical tool is created. A researcher can take an organic solution, inject it into the instrument, separate the individual components, and identify each of them. Furthermore, the researcher can determine the quantities (concentrations) of each of the components after careful calibration.

Phenethyl alcohol (85-216 ppm) and isoamyl alcohol (38-115 ppm) constituted the majority of fusel oils.

The analysis was carried out on an Agilent 7890 GC equipped with an Agilent 5975C inert MSD with triple Axis Detector. The temperature of inlet heater was 230^0C. The separations were performed using an Agilent 190915-433 (30m×250µm×0.25µm) column with an oven temperature 32^0C to maximum 320^0C. The programmed used was 32^0C for (5minute) up to 230^0C with 5^0C per minute then hold time 15 minutes, which took total 59.6 minutes. The carrier gas was helium with flow rate of 1ml/minute. The electron impact energy (ionization voltage) was 70eV, 2µL of sample was injected with split ratio 75:1.

CHAPTER 4

RESULTS AND DISCUSSIONS

Wine from rice is produced after saccharification of starch by microbes, enzymes (especially, commercial amylase) followed by alcoholic fermentation using yeasts. The rice wine quality differs with rice varieties, strains of yeast, mold, processing technique, aging, etc. Murcha is the indigenous starter culture used for the preparation of jand and raksi; and is the source of different kinds of mold, yeast and bacteria (Dahal et.al 2012). In this research, mold was isolated from murcha collected from different parts of Nepal. Then, the molds were identified by analyzing their morphological characters. The mold having high saccharifiying ability was selected and used for the rice wine production.

4.1. Identification of molds in different *murcha* sample

Murcha sample were collected from different parts of Nepal. The murcha were different in colour due to different types of flour (such as wheat, corn, millet, rice etc) used in the preparation. The collected murcha samples were grinded and one gram of each murcha sample was spread in PDA media plates containing 50 ppm Chloramphenicol. After 4 to 5 days of incubation, molds colonies present in murcha samples were observed in the PDA plates (see Figure 4.1). The observed morphological characteristics of molds are given in Table 4.1.

Table 4.1 Morphological characteristic of isolated mods.

S.N.	*Murcha* code	Number of colony/gram *murcha* sample	Colony color	Colony diameter (mm)	Conidia shape	Conidia surface
1	Rm	4	Greenish yellow	55	Ellipsoid	Rough
2	Sm	3	Greenish yellow	50	Spherical	Smooth
3	Dm	6	Creamy white	60	Spherical	Irregular
4	Tm	4	Light yellow	48	Columns	Rough
5	Pm	5	Creamy white	50	Spherical	Irregular
6	Lm	4	Cottony white	45	Spherical	Rough
7	Bm	3	Light yellow	47	Columnar	Rough

*Note: Rm = Rajbiraj murcha, Sm= Siraha murcha, Dm= Dharan murcha, Tm= tamaghas murcha, Pm= Phidim murcha, Lm= Lalitpur murcha, and Bm= Bhaktapur murcha

Table 4.1 showed that the maximum number of mold colony per gram murcha was observed in the murcha sample collected from Dharan. As observed the colony were

greenish yellow, light yellow or creamy white. The size of mold colonies were in the range of 45 to 60 mm. The conidia was spherical, columnar or glubose shape with smooth, rough or irregular surfaces. The morphological characteristics of the isolated molds were similar to Aspergillus species (Afzal *et al*., 2013).

4.2 Selection criteria of molds

4.2.1 Starch hydrolysis

The result of iodine test of isolated molds for starch hydrolysis showed clear transparent zone of hydrolysis on the PBSA media plate. Among the seven different isolated mold colonies, all the colonies formed clear transparent zone which is shown in Figure 4.2. Formation of this clear zone around the mold colony indicated that the molds can show amylolytic activity.

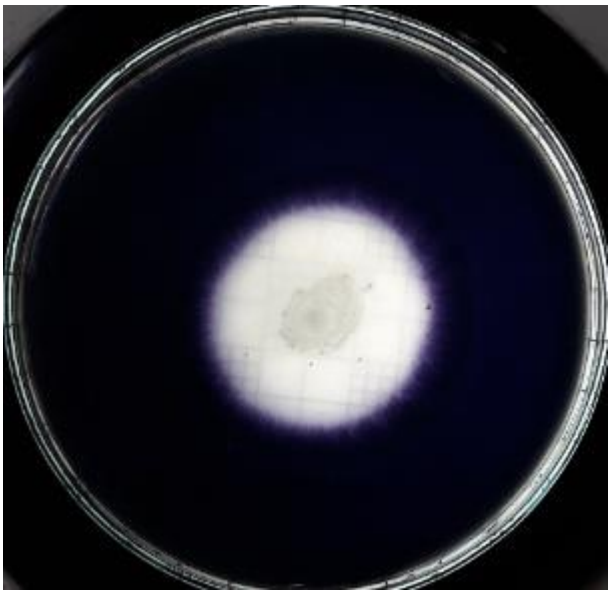

Figure 4.2 Zone of hydrolysis in PBSA plate

The mold isolated from Rajbiraj murcha produce clear and larger halo zone than rest of isolates. 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 & Abakaliki, 2013).

Some species of mold produce an enzyme complex which both dextrinized and saccharified starch at rapid rate, these enzymes are similar to enzymes of amylolytic bacteria.

Aspergillus species mainly produce dextrinizing enzyme, which is similar to the α-amylase produced by the malt. In place of β-amylase the dextrinizing enzyme of saccharifying mold is complemented by maltase.

4.2.2 Detection of non-toxigenic (aflatoxin) molds

Non-toxigenic mold was detected based on two methods:

a) Detection of non-toxigenic mold based on colony fluorescence

The isolated molds are sub-cultured in CMA media and incubated at 28 ^{0}C for 3 days and plates are visualized under 350nm UV radiation. No fluorescence seen under UV radiation, which indicate that all the isolated molds are non-toxigenic. Most of the aspergillus species produce different kinds of aflatoxin, and have similar morphological structure, and this makes difficult to distinguish between them. Detection of non-toxigenic mold based on the fluorescence of colonies upon exposure to UV radiation at 365nm. When expose to UV light, aflatoxin producing mold produce blue fluorescence on the undersides of the colonies, whereas the non-producing isolates did not produce any fluorescence. In this study isolates molds were not produce blue fluorescence on the undersides of the colonies on exposure to UV radiation. This is easiest and fastest technique for distinguishing toxigenic and non-toxigenic molds (Thathana *et al*., 2017).

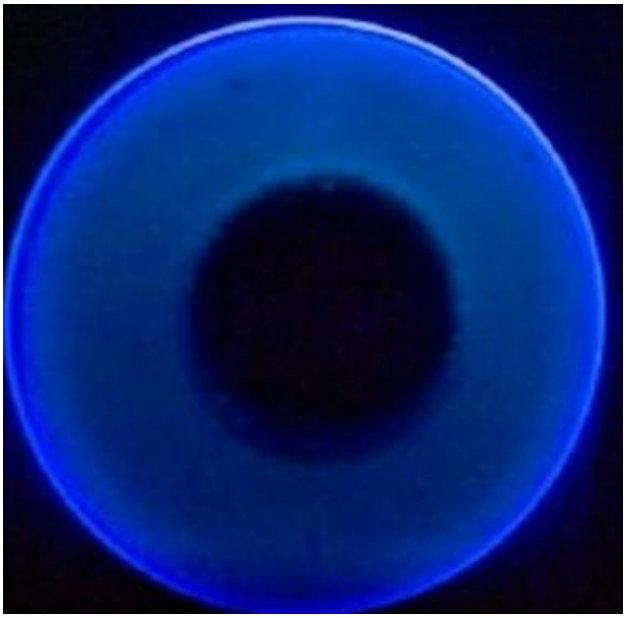

Figure 4.3 CMA plate with fungal colony showing no fluorescence on UV exposure

b) Ammonium hydroxide Vapor-Induced color change

No plum-red color was observed which indicates that the isolated molds are non-toxigenic molds. Ammonium hydroxide vapor method is rapid and effective method for aflatoxin detection. In this method the aflatoxin producing mold produce plum-red color on exposure to ammonium hydroxide vapor. None of the isolates produces plum-red

colour on exposure to ammonium hydroxide vapour. Which indicates that the isolated molds are non-toxigenic molds. Aflatoxin producing mold produce aspergillic acid which on reaction with ammonium hydroxide vapor produce plum-red color (Variane *et al*., 2018).

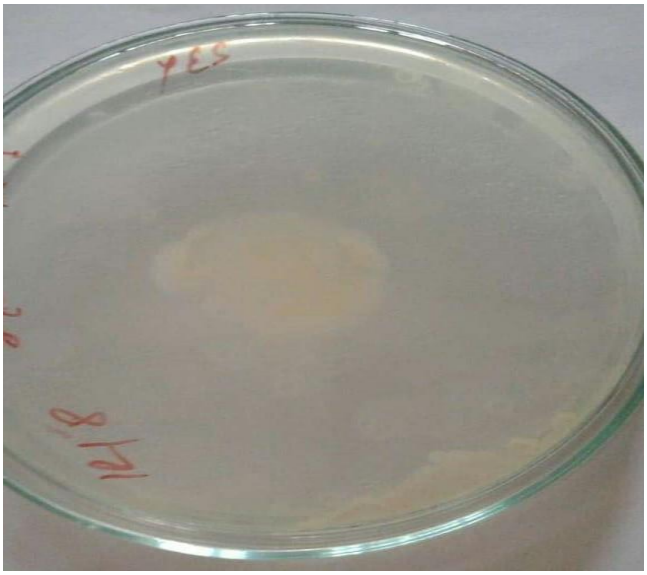

Figure 4.4 No characteristic plum red color change on reverse side of YES plate.

There are several factors which affect the production of aflatoxin such as temperature, moisture, pH, media combination, incubation time and inoculum size. Media combination provide great advantage on detection of aflatoxin producing mold. Combined use of CYA and YES media is advantageous since both of these media allow to produce different kinds of aflatoxins (Kayalvizhi & Antony, 2011).

4.3 Saccharification test of different mold isolates

The amount of reducing sugar released was measured by the DNS method and percentage of saccharification was calculated by applying the equation of Spano et al. All the isolated molds showed good result. The mold isolated from Rajbiraj murcha showed better saccharification than other molds isolates. Saccharification rate was increased with increase in incubation time, the highest percentage of saccharification was shown 96-hour incubation. DNS test showed that there was significant amount of reducing sugar produced during saccharification test. The mold isolated from Rajbiraj murcha produce higher amount of saccharifying enzyme than other isolates.

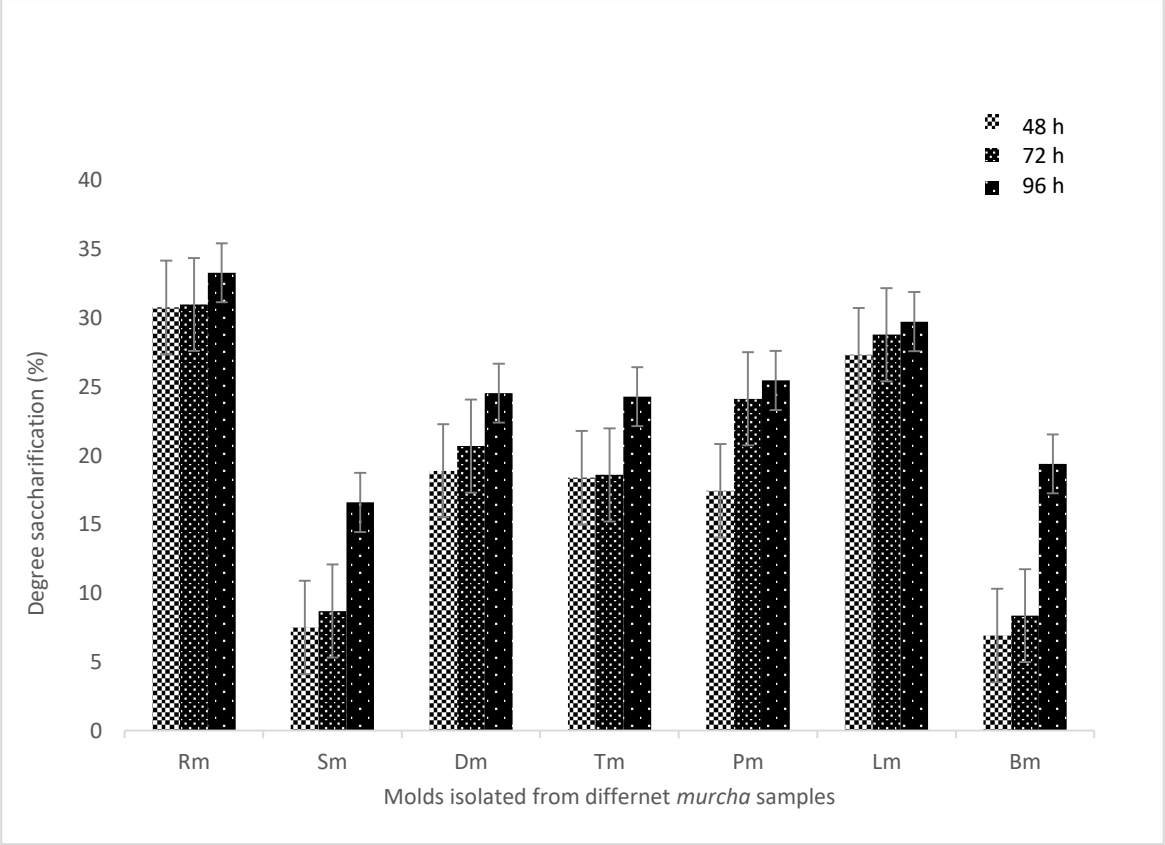

Figure 4.5 Saccharification percentage of isolated molds at different incubation time. Vertical bars indicate ± standard error.

Rm= Rajbiraj *murcha*, Sm= Siraha *murcah*, DM= Dharan *murcha,* Tm= Tamghas *murcha*, Pm= Phidim *murcha*, Lm= Lalitpur *murcha*, Bm= Bhaktapur *murcha*

Production of saccharifying enzymes depends on the moisture content of the substrate, high moisture content results in decrease the porosity of substrate and reduce the oxygen penetration on other hand decrease the growth of mold (Aggarwal *et al.*, 2017).

The activity of enzyme depends on time, temperature, pH and enzyme source. Increase in temperature leads to increase in enzyme activity but also accelerate the denaturation by higher physiological temperature. In general enzymes are less stable over time at elevated temperature at pH value near the optimum limit. 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 (John Mathew *et al*., 2016).

4.4 Rice wine production from selected mold isolate and yeast and murcha

The inoculum for fermentation were prepared in YEPD broth and PBS broth of commercial yeast and mold of Rajbiraj murcha. Mold isolate was used to produce koji-mold for rice wine production. Fermentation was done in 2 different jar one in one jar the murcha was used as starter culture and in another jar isolated mold and commercial

yeast was used as starter culture. Fermentation was done up to 16 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.

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 by activated charcoal.

4.5 Changes in ˚Brix during fermentation

During 16 days of rice mash fermentation at room temperature (20±2˚C). Change in ˚Brix, was calculated Change in TSS (^{0}Brix) during fermentation of rice wine by using two different starter culture (mold isolated from Rajbiraj *murcha* and yeast and *murcha*) up to 16 days of fermentation at room temperature was obtained as shown in figure below. Continuous increase in ˚Brix of rice mash from upto day 6 and after day 6. And decrease in ˚Brix. After day 10 of fermentation there was no change in ˚Brix.

At the initial stage of fermentation ˚Brix gradually start to increase. ˚Brix was increase up to 6 days of fermentation after that it was starts to decrease and after 10 days of fermentation it remains constant.

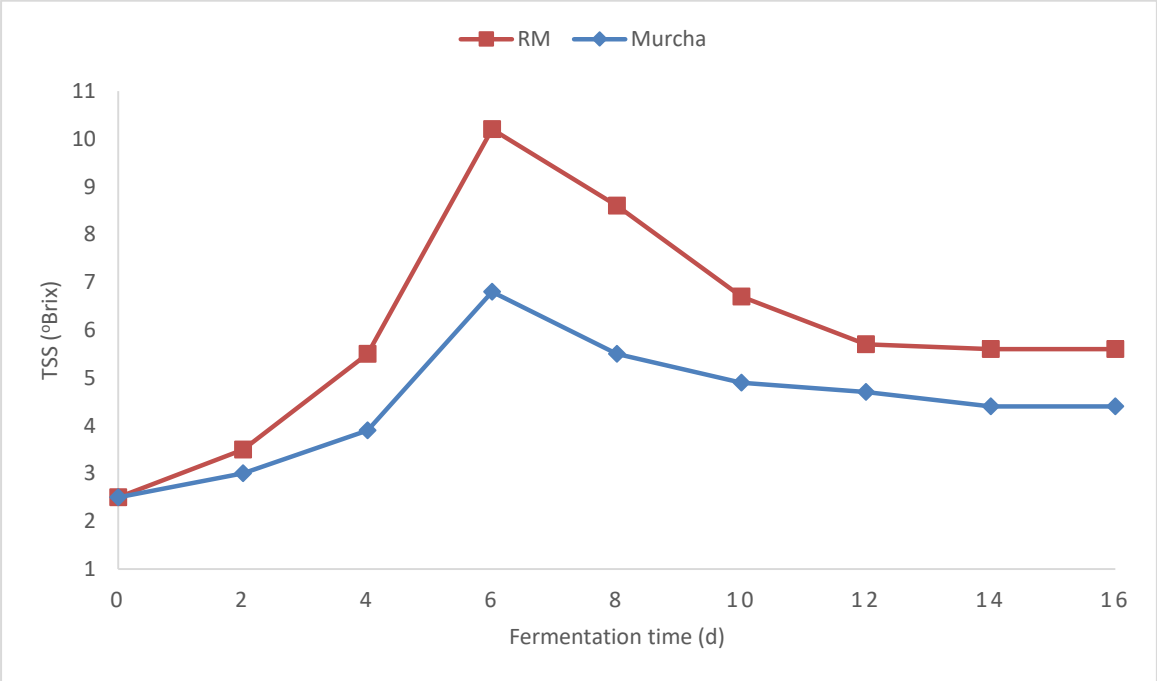

Figure 4.6 Change of in TSS (^{o}Brix) during fermentation

Continuous increase in ˚Brix may be continuous inoculation of yeast, koji and steam rice in fermentation tank during day 2, day 4 and day 6 of fermentation and also less activity

of yeast strain to produce ethanol as compared to fermentable sugar produce by mold. After 8 days of fermentation gradually decrease in ˚Brix indicated that the yeasts start to ferment the glucose produced by saccharification of starch of rice and then after 10 days fermentation almost ceased that might be due to concentration of ethanol or very less power of saccharificaton by mold. Chay et al., 2017 reported that finally change in ˚Brix stop at 5 ºBrix during rice wine preparation.

4.6 Change in pH during fermentation

The pH change was measured with pH meter, an instrument that determines pH quickly and easily. Gradually decrease in pH is observed with increase in fermentation time. The rice wine which is made by *murcha* sample shows lower pH than that of wine prepared by isolated mold and yest.

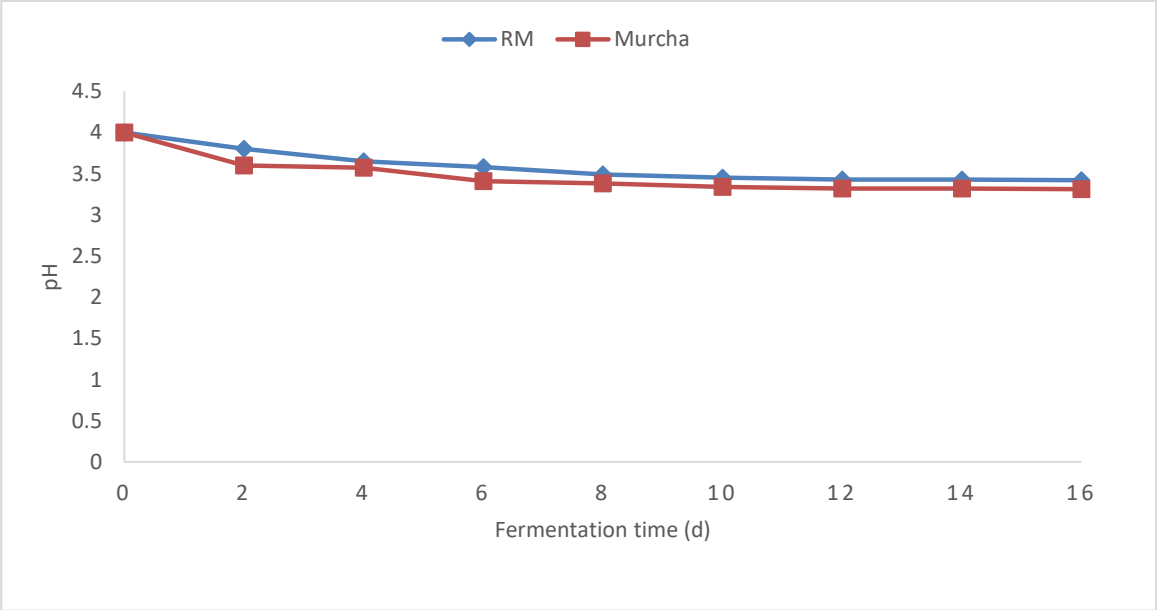

Figure 4.7 Change in pH during fermentation of rice mash at room temperature (20±2˚C)

In this study initial pH of fermentative mash was 4, so there was no addition of any acid to maintain the pH that might be due to acidic nature of koji caused by growth of LBA during preparation and storage of koji. According to Harandi et al., (2017) pH=4 provided the best condition for yeast cells to reproduce and grow. The pH of the broth as it is one of the key factors for ethanol production having direct influence on organisms as well as on their cellular processes.

There was decrease in pH up to 4^{th} days of fermentation, that might be due to the production of ethanol and CO_2 during fermentation. CO_2 is one of the key factor which reduce the pH of fermentative broth and production of organic acids like succinic acid and pyruvic acid (Chay *et al.*, 2017).

4.7 Changes in acidity during fermentation

The titrable acidy of two wines gradually increasing along with fermentation time. The titrable acidity of rice wine which is made up of by using isolated mold and commercial yeast shows lower acidity than that of wine which made up of *murcha*. After few days the acidity of wine which is made up of by using isolated mold and yeast remains constant but rice wine made by murcha was increasing till last day of fermentation.

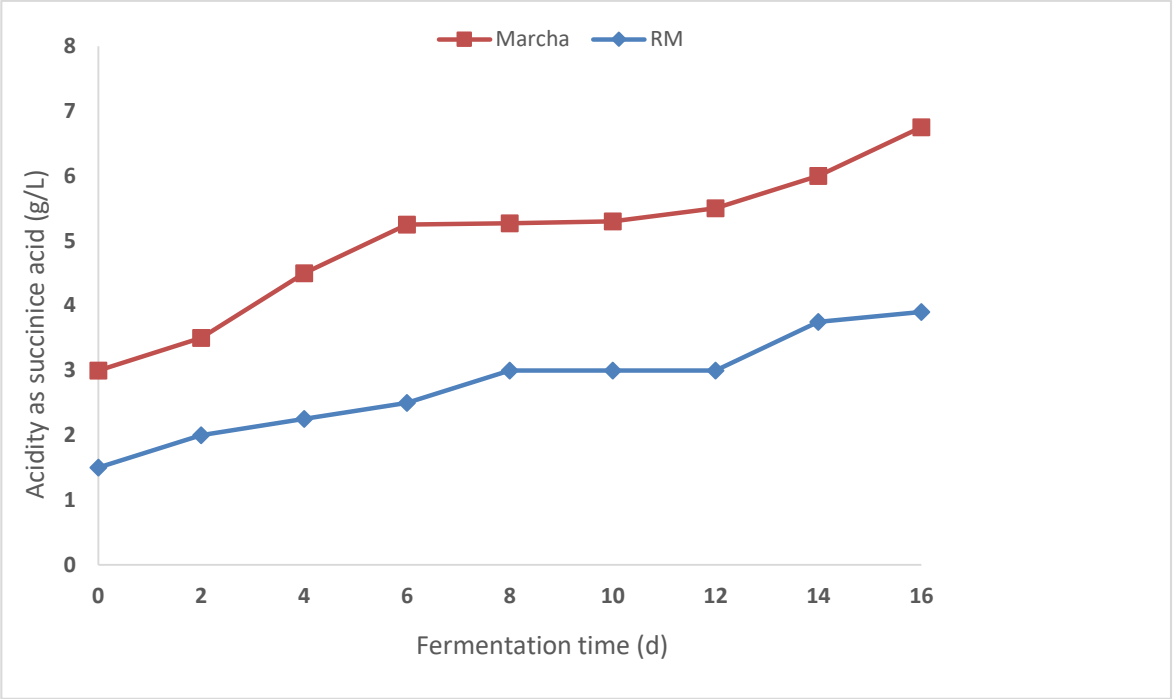

Figure 4.8 Change in acidity during fermentation of rice mash at room temperature (20±2°C)

There was increase in acidity in initial days of fermentation, may be due to formation of different kinds of organic acid and CO_2 during fermentation. During initial and mid period of fermentation process there were 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. The organic acids identifed in rice wine during fermentation were oxalic acid, tartaric acid, pyruvic acid, malic acid, α-ketoglutaric acid, lactic acid, citric acid and succinic acid (Xu *et al*., 2018).

According to yeast fermentation theory, via glycolysis process yeast cell ultimately degrade glucose to pyruvic acid will be converted to alcohol under non-oxidative environment. During fermentation process glucose also undergo other method such as sugar degradation to produce organic acids (Jia *et al*., 2019). There for during fermentation of rice wine, the total acid content gradually increased, as the fermentation time prolonged, sugar in fermentation broth were completely

decomposed and acidity was maintained. According to (Bhatane & Pawar, 2013) 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.

4.7 Comparative study of Lab prepared sake with commercially available sake

Comparative study of lab prepared *sake* and commercially available *sake* has been done. There is slightly different in parameters in lab prepared sake than that of commercially available *sake*. The alcohol content, ester, ˚Brix, and pH are higher than the sake prepared in lab. Methanol was not present in commercial *sake*.

Table 4.7 Lab prepared *sake* and commercial *sake* are compared for their physio-chemical properties

S.N.	Particles	Rm	Crude *Murcha*	commercial sake
1	Alcohol (%Abv)	8	6	15
2	pH	3.42	3.31	4.7
3	°Brix	5.6	4.4	6.3
4	Succinic acid (g/L)	3.9	10.09	5
5	Methanol% (v/v)	0.019	0.021	0
6	Ester (mg/L)	11.1	11.7	30

Rice wine produced by isolated mold and yeast from marcha sample after 16 days of fermentation at room temperature (20±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 Rajbiraj marcha, different chemical parameter of rice wine was calculated. But ethanol (%abv), and ˚Brix were lower than that of commercial rice wine. That may be due to following reasons:

- Rice variety used was different than commercial rice wine variety
- Less policing of rice.
- Fluctuation of fermentation temperature.
- During fermentation lab yeast was used instead of sake yeast.

Quality of rice wine differs with rice variety different rice variety and fermentation method, significant differences are observed with respect to various parameter like temperature, pH, titratable acidity, total soluble solid, alcohol contents, protein and

reducing sugar and overall sensory acceptance (Woldemariam et al., 2014). 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 (Liu *et al*., 2016).

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%. Rice wine made using polished rice at 60 and 70% had higher concentration of ethyl acetate and isoamyl alcohol, that contribute the overall aroma of rice wine. Polished at 60 and 70% contribute more to quality of rice wine than polished at 80 and 90% (Arachchige *et al*.,2011).

4.8 Determination of Essential Oils by GC/MS

The chromatogram Figure 4.9.1 indicates that there are basically 6 major peaks indicating the respective compounds. The sample injected contains acetic acid as major compound that contain 37.05% (Retention time 2.398). While second major compounds have been predicted to be either propanoic acid, 2- Hydroxyl ethyl ester or, Ethanol 2 2-oxybis (retention time 6.997 minutes). Similarly, another major compound has been predicted as 2 hydroxy propanoic acid or formamide (retention time 11.170 minutes). While other minor compound with smaller peak have been also detected such as Phenyl Ethyl alcohol (retention time17.564 minutes), Glycerin or 4-Hydroxy-3-[[1,3-dihydoxy-2-propoxy] methyl]-1H-pyrazole-5-carboxomide (retention time14.514 minutes) and 1-Butanol-3-methyl or, 1- Pentanol (rentention time 4.116 minutes).

The chromatogram Figure 4.9.2 concludes a ten different peaks indicating ten different compounds. It contains 1-Butanol, 3 methyl as major compound which constitutes 20.07% (retention time 4.138 minutes), similarly second major compound has been predicted as either Formamide or Propanoic acid or, 2-hydroxy ethyl ester which constitutes 13.38%with retention time 7.018 minutes. From another distinct peaks, Phenyl ethyl alcohol has been predicted which constitutes 11.95% with retention time 17.569 minutes. Different minor compounds has been predicted with smaller peaks in which 2-[2-Hydroxyethyl]-9-[beta-d-ribofuranosyl]hypoxantine or 1- propanol or 2-methyl with 9.21% (retention time 2.218 minutes). Similarly another minor compound has been predicted as Cyclopentane,1,2-dimethyl or Cis 2H-pyran-2,6(34)-dione or dihydro-4 or 4-dimethyl or 3,3- Diethyldiaziridine with 4.69% (retention time 4.252). From another small peaks, Dimethyl-cyanophosphine or 2,3-Butanediol has been

predicted 3.34% with retention time 6.195 minutes. Similarly another minor compounds has been predicted as Ala-gly or, trimethylsilylester or 1,2-Benzenediol or 4-[2-(methylamino) ethyl] or di-alpha- (Methylaminomethyl) benzyl alcohol with 2.94% concentration and 37.410 retention time. And smallest peak indicates the minor compound as propanic acid, 2-caminoxyl, Acetic acid or cyclohexan-1,4,5-triol-3-one-1-carboxylic acid with 2.79% at 2.163 minutes retention time.

Results were found to be shown below as graph:

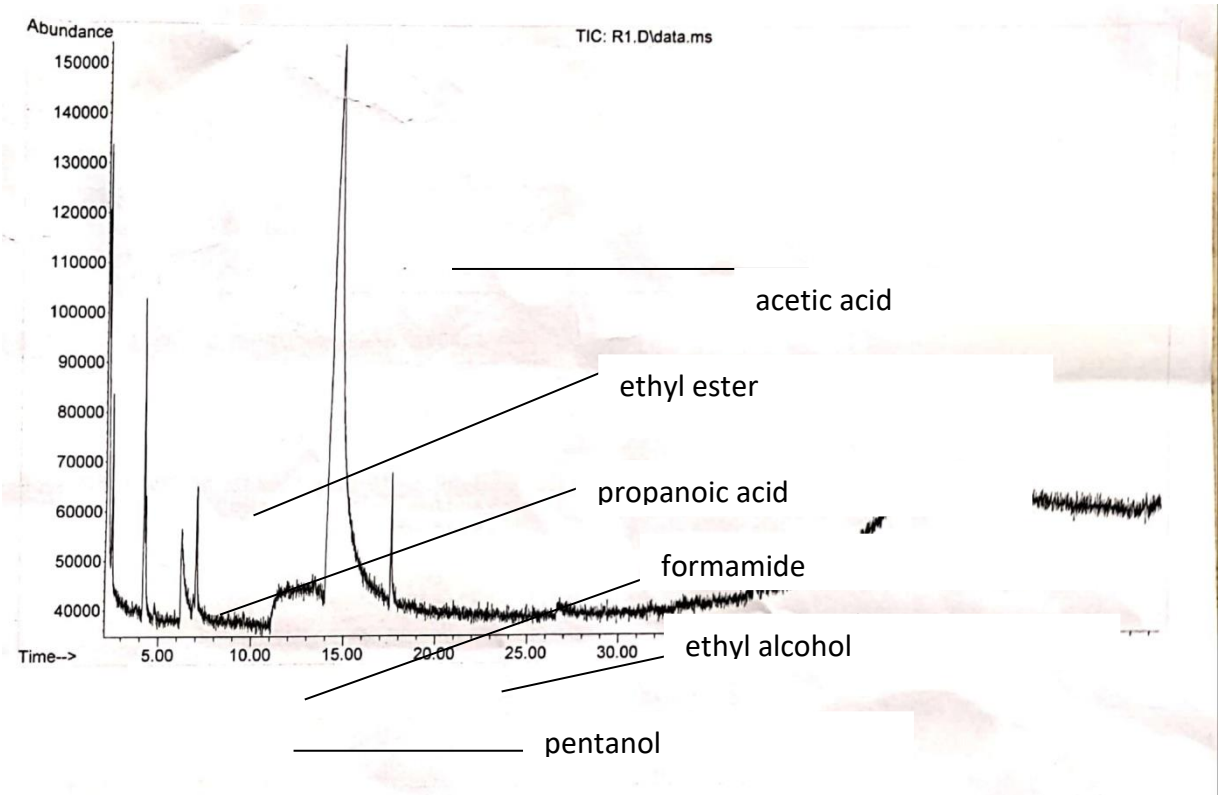

Figure 4.9.1 Rice wine's GC/MS picture by using acq method for essential oil (Abundance vs Time)

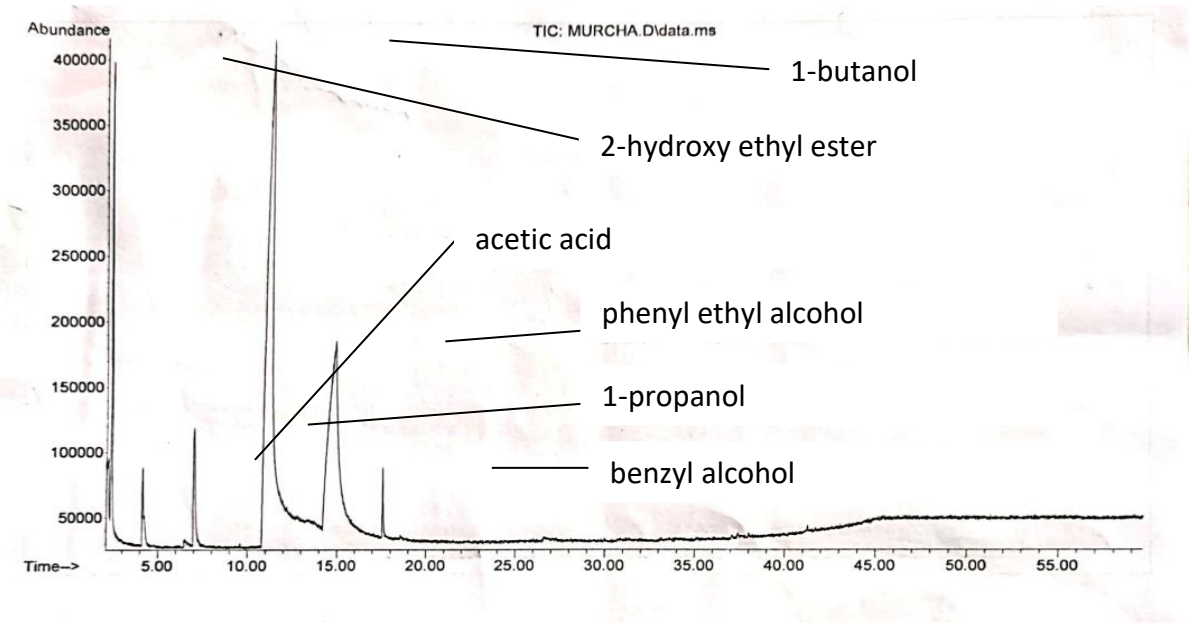

Figure 4.9.2 Crude *Murcha* wine's GC/MS peaks by using acq method for essential oil (Abundance vs Time)

The fusel alcohols in the traditional alcoholic beverage are mainly formed by the fermentation of the amino acids in the yeast during the fermentation process. The amount produced differs according to the type of yeast, fermentation temperature, oxygen concentration, degree of agitation, and amino acid content in the raw material rice. According to previous studies, alcoholic beverage contains n-propyl alcohol and n-butyl alcohol that are formed through α-ketobutyric acid by the Enrlich pathway. Isobutyl alcohol that is formed by valine, and isoamyl alcohol that is formed by leucine and carries the sweet aroma of banana, are also present. Another component of this beverage is phenethyl alcohol, which has the scent of rose and honey and is found in natural oils such as rose petals and orange blossoms. Phenethyl alcohol is formed by phenylalanine and is one of the most important aromatic alcohol compounds in beer.

CHAPTER 5

SUMMARY

In this study, seven different isolates of molds isolated and preserved. All the isolates showed similar morphological characteristics with *Aspergillus* species. Further used for determining their non-toxigenic property, the non-toxigenic properties were determined by two method, one is based on colony fluorescence and another is ammonium hydroxide vapour induced color change. In colony fluorescence method the molds were gwon on CMA media plate for 3 to 4 days at 28^{o}C and viewed under UV at 350nm. All the isolates showed negate result. Another method is ammonium hydroxide vapour induced colour change. In this method the isolates were grown in YES media plate for 3 to 4 days at 28^{o}C and ammonium hydroxide was poured. All the isolates showed negative result. Which indicates the isolated molds are no-toxigenic. And then saccharifying capacity of the molds were measured. The mold which has higher saccharifying capacity was selected for *sake* preparation. Among these seven isolates molds, the mold isolated from Rajbiraj *murcha* showed maximum percentage of saccharification and Liquification.

The rice wine produced by isolated mold from Rajbiraj *murcha* and yeast was found to be 8% ABV total acidity in terms of succinic acid (g/L);3.9, ^{o}Brix 5.6, pH, 3.42, ester (mg/L); 11.1, and methanol(V/V) 0.019 for pasteurized rice wine. It was compared with commercial and traditionally prepared rice wine based on physiochemical properties. The lab prepared rice wine had similar quality parameters as commercial rice wine and traditionally prepared rice wine. But the commercial rice wine had higher alcohol (17% Abv) than that of lab prepared rice wine (8% abv) and traditionally prepared rice wine had (6% Abv). Also the ^{o}Brix 6.6 is slightly higher in commercial rice wine than lab prepared one and traditionally prepared one. The lab prepared rice wine had ^{o}Brix 5.6 and traditionally prepared rice wine had ^{o}Brix 4.4.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The conclusions of this study are as follows:

a) Murcha sample collected from Rajbiraj had highest percentage of saccharification and liquification of steamed rice among all murcha sample.
b) The collected murcha samples didn't have any types of aflatoxin producing molds.
c) Low calorie rice wine can be produced by using local raw materials.
d) There was similarities in alcohol content and other component like taste and flavour, between lab prepared rice wine and traditionally prepared rice wine.

6.2 Recommendations

Since the concentration of ethanol is lower than the commercial rice wine, during fermentation of rice wine by using local raw materials. Hence, for the further work, following suggestion is recommended.

a) Mold and yeast from the experiment produced low alcohol content (% abv). These mold and yeast are not suitable producing higher alcoholic rice wine.
b) Different varieties of rice can be affect quality of the final product.
c) Degree of polishing of rice can be varied to optimize better quality rice wine.
d) Aging should be done for better quality of rice wine.

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APPENDIX

Appendix I

Table 3.2

Composition of YPD agar media	
YPD Agar	Ingredients(g/L)
Yeast extracts	3
Peptone	5
Glucose	10
Agar	20
Distilled water	1

Table 3.3

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

Table 3.4

Composition of Czapek-dox agar media	
CYA Agar	Ingredients (g/L)
Sucrose	30.0
Sodium Nitrate	2.0
Dipotassium phosphate	1.0
Magnesium sulphate	0.5
Potassium chloride	0.5
Ferrous sulfate	0.01

Agar	15.0
Yeast extract	5
Distilled water	1

Table 3.5

Composition of YES agar media	
YES Agar	Ingredients (g/L)
Yeast extract	4.0
Sucrose	20.0
KH_2PO_4	1.0
$MgSO_4$	0.5
Agar	15
Distilled water	1

Table 3.6

Composition of Starch agar plate media	
SAP media	Ingredients (g/L)
Beef extract	3.0
Soluble Starch	10.0
Agar	12.0
Pepton	5.0
Distilled water	1.0

Appendix II

Figure 3.1 Cooling and adding yeast

Figure 3.2 Sake before and after filtration

Figure 3.4 Filtration by using char

Figure 3.5 Ageing product

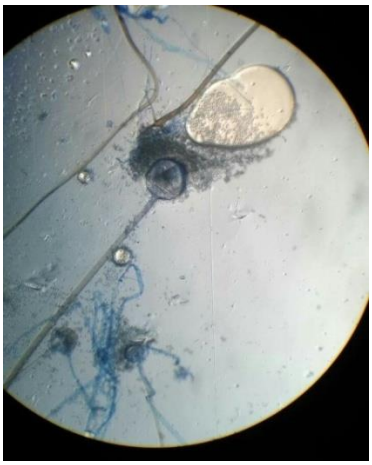

Figure 4.1 Microscopic examination of spore of mycelium