PREPARATION AND QUALITY EVALUATION OF SORGHUM *JAND* BY USING DEFINED FERMENTATION STARTER UNDER SOLID AND SEMI-SOLID STATE FERMENTATION

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Preparation and Quality Evaluation of Sorghum *Jand* by Using Defined Fermentation Starter under Solid and Semi-solid State Fermentation

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Approval Letter

This ***dissertation*** **entitled** ***Preparation and Quality Evaluation of Sorghum Jand by Using Defined Fermentation Starter under Solid and Semi-solid State Fermentation*** **presented by Saurav Shrestha has been accepted as the partial fulfillment of the requirements for the B.Tech. degree in Food Technology**

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Abstract

Jand was prepared from sorghum using traditional and pure fermentation starters under solid and semi-solid fermentation states with 50, 75 and 100% water addition. Saccharifying molds (identified to be *Rhizopus* spp.) and fermenting yeasts (identified to be strains of *Saccharomyces cerevisiae*) isolated from market *murcha* were used to make pure-cultured fermentation starter and the *Jand* was subjected for sensory and chemical analysis of total acidity, pH, alcohol content, dry matter content, esters content, aldehydes content, reducing sugars and protein solubility.

Raw sorghum had moisture content, crude fat, crude protein, crude fiber, ash content and starch content of 10±1.24%, 2.367±0.2%, 13.39±0.6%, 1.93±0.4%, 1.67±0.4% and 58.57±4% respectively. The solubility of protein in sorghum extracted at pH 5.2±0.1 was found to be 28.4±1.5%. Results indicated that except on dry matter content, use of pure culture had significant effect ($p<0.05$) on pH, alcohol content, acidity, esters, aldehydes, reducing sugar, methanol and protein solubility in solid and semi-solid states. Sensory attributes (flavor, mouth feel and overall acceptability) of *Jand* from two types of *murcha* were found significantly different with each other ($p<0.05$) in solid state. While, results indicated that except on aldehyde, alcohol, dry matter contents and reducing sugar, all fermentation states were significantly different ($p<0.05$) with each other in terms of total acidity, pH, esters content, methanol content and protein solubility. Increasing the folds of water addition in semi-solid state fermentation caused remarkable increment in acidity, with nearly of 2 times than that of the solid state, but had no remarkable improvement on the sensory quality of sorghum *Jand.* Use of pure culture and increasing water addition in semi-solid state fermentation increased protein solubility of sorghum nearly upto 58%.

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List of abbreviations

Abbreviations	Full form
MYGP	Malt Yeast extract Glucose Peptone
ml	Milliliter
G	Gram
ºC	Degree Celsius
ºBx	Degree Brix
ANOVA	Analysis Of Variance
Fig.	Figure
m/m	Mass by mass
m/v	Mass by volume
v/v	Volume by volume
Lit.	Liter
Hrs.	Hours
Wt.	Weight
LAB	Lactic Acid Bacteria
db	Dry basis
RH	Relative Humidity
LSD	Least Significant Difference

Part I

Introduction

1.1 General Introduction

Nepal is rich in food and cultural biodiversity. People in Nepal are engaged in agriculture practices and produce different cereals, grain, vegetables, tubers and fruits. People are more concerned at preparing and preserving locally available foods and food security for a very long time (T. B. Karki *et al.*, 2005). People have been using different methods to utilize the agricultural products as well as to preserve for long time. But no attempts have been made in the utilization and preservation of cereal sorghum due to its low digestibility in both protein and starch (Zhu, 2014). The nutritional properties of sorghum are unique and variety dependent.

Sorghum is gluten-free, thus can be consumed by people with celiac disease (Taylor *et al.*, 2006). It is noted for the relatively low digestibility of both protein and starch with great potential for weight and obesity management. Some varieties are rich in polyphenols, especially condensed tannins, as natural antioxidants (Dykes and Rooney, 2006). Other important nutrients of sorghum include dietary fiber, fat-soluble and B-vitamins, and minerals (Waniska *et al.*, 2004). All these nutritional properties make sorghum gain a spotlight for better production and utilization as human food in various forms. Sorghum has also been an important feed for ruminants, pigs, and poultry.

Various strategies have been suggested to improve feed values by increasing the digestibility and solubility of protein and solubility of starch (Liu *et al.*, 2013). Among them, fermentation of cereals using starter cultures has been found to be advantageous for several reasons (Harlander, 1992; Hesseltine, 1992; Nout, 1992). Many of the starters are impressively durable (Hesseltine *et al.*, 1988; Karki, 1986; Nout, 1992). The most notable advantage of using starter cultures is the simultaneous saccharification, souring and fermentation due to the presence of molds, yeasts, and lactic acid bacteria. This feature makes unnecessary the use of enzyme source which can help in increasing starch digestibility, protein digestibility and solubility of cereals especially sorghum and food fermentation is regarded as one of the oldest methods of food processing and preservation as well (Achi, 2005).

Among the fermented beverage, *Jand* is indigenous to Nepal which is prepared by solid-state fermentation of starchy materials using *Murcha* (a traditional fermentation starter). Although the technology of *Jand* making has been practiced since antiquity, its production is still limited to home scale in Nepal. It has many shortcomings as a result of which the final product quality is the mercy of the *Murcha* and environmental conditions used during processing. It has a great potential of being commercialized, both in domestic and foreign markets, provided its quality (safety and nutritional value) is enhanced, which calls for a sound research and development work. Much literature is available on the traditional alcoholic fermentation of starchy raw materials under solid-state conditions using both traditional as well as defined fermentation starters (starters prepared by using pure microbial cultures isolated from traditional starters (Bhandari, 1997; Rajbhandari, 1999; Subba, 1985; Upadhyaya, 2005; Venkataramu and Basappa, 1993; Verma, 1991) but information regarding semi-solid fermentation is scanty (Cai and Nip, 1990; Dung *et al.*, 2007).

In Nepal, all traditional cereal-based alcoholic beverages are produced under solid-state fermentation and, hence this work was undertaken aimed at investigating the effect of semi-solid fermentation on the quality of sorghum *Jand* (millet beer) using defined fermentation starter prepared from *R. oryzae* and *S. cerevisiae*. The combined study of the effect of fermentation states being solid and semi-solid and use of defined as well as traditional cultures on the quality of sorghum *Jand* would help in the scope of utilization of sorghum in alcoholic beverages, scope of investigation on semi-solid fermentation and scope of improvement in protein digestibility as well as solubility in sorghum.

1.2 Statement of the problem

More than two-thirds of the workforce in Nepal is engaged in agriculture and a considerable percentage of the total populations are living below the poverty line. Sorghum is generally associated with low-income communities in Nepal and it is well adapted to grow in dryer climates and tolerates hot climates better than maize or soybeans. It is a warm season grass and has adapted to grow in a wide range of soil types often tolerating waterlogging and many poor soil types. Despite of the fact, sorghum is an underutilized crop, somewhat forgotten by Nepalese society due to lack of knowledge about its nutritional aspects and low protein and starch digestibility as well as complex starch-

protein interactions and presence of inhibiting factors in the grain (Hamaker *et al.*, 1986; MacLean *et al.*, 1981; Mertz *et al.*, 1984).

Fermentation causes significant reduction in trypsin and amylase inhibitors activities and the phytic acid content (Osman, 2011) which can lead to ultimate increment in protein digestibility and solubility in the cereal. For this, *Jand* can be valuable in modifying chemical properties of sorghum, as *Jand* is Nepalese indigenous fermented beverage. But, although, the technology of *Jand* making has been practiced since antiquity, its production is still limited to home scale in Nepal. Besides this, in Nepal all traditional cereal-based alcoholic beverages are produced under solid-state fermentation, but there is lack of information about use of semi-solid fermentation state and its effect on the sensory and chemical properties of a grain. The functional properties of sorghum proteins can be used to define how sorghum can be used to supplement or replace more toxic protein sources (e.g. wheat). There is, however, no information on the functional properties of fermented sorghum.

Above the all, *murcha,* which is used as traditional starter in making fermented products has its trade reflecting an important economic activity for many ethnic groups of Nepal, as this is the sole source of income for many of them. However, the tribal method of *murcha* preparation is very primitive and often prone to failure. Review on amylolytic starter cultures by (Nout, 1992) shows distinct possibility for the development of traditional starters into commercial forms that are safe, efficient, and stable which may also help in upgrading sensory and chemical properties of the cereal. The information about the above mentioned concerns is essential for determining potential uses of this sorghum in food formulations and alcoholic beverages.

1.3 Objective

1.3.1 General objective

Preparation and quality evaluation of sorghum (*S. bicolor L. Moench*) *Jand* by using defined fermentation starter under solid and semi-solid state fermentation.

1.3.2 Specific objectives

- Isolation of yeasts and molds from *murcha.*
- Selection of mold based on their saccharification activity.
- Preparation of koji from selected molds.
- Preparation of *murcha* from pure culture.
- Preparation of *Jand* from sorghum using pure and traditional murcha under similar environment of solid and semi-solid state fermentation.
- Comparison of sensory and chemical properties of *Jand* prepared from pure and traditional murcha under solid and semi-solid state fermentation.
- Comparison of protein solubility of sorghum before and after fermentation.

1.4 Significance of study

Sorghum *(Sorghum bicolor L. moench)* is the 5th most important cereal globally in terms of acreage and production (Beta *et al.*, 2004; Waniska *et al.*, 2004). Sorghum has great drought-tolerance and requires minimal fertilizers on marginal lands for cultivation, thus playing a critical role for food security in some semiarid areas of Asia, Africa, and Latin America (Beta *et al.*, 2004; Dicko *et al.*, 2006). The traits of low–input cost and adaptability to a wide range of environments make sorghum a favorable potential candidate for various food and nonfood products. The nutritional properties of sorghum are unique and variety dependent. Sorghum is gluten-free, thus can be consumed by people with celiac disease (Taylor *et al.*, 2006).

It is noted for the relatively low digestibility and solubility of both protein and starch with great potential for weight and obesity management. Some varieties are rich in polyphenols, especially condensed tannins, as natural antioxidants (Dykes and Rooney, 2006). Various strategies have been suggested to improve feed values by increasing the digestibility and solubility of both protein and starch (Liu *et al.*, 2013) in which

fermentation can be one of them which can be studied in both solid and semi-solid state fermentation. This would lead to the study of effects of semi-solid state fermentation in comparison to solid state and to study the changes in sorghum as well as its fermented products in terms of sensory as well as chemical properties point of view.

Thus, this dissertation work is directed to study chemical changes in sorghum by the parallel study of solid and semi-solid state fermentation of the cereal. Besides this, development of starter culture from pure cultures will help in discouraging the dependence on spontaneous fermentation. Identifying and providing a practical means of using appropriate starter cultures is advantageous due to the competitive role of microorganisms and their metabolites in preventing growth and metabolisms of unwanted microorganisms. A strong starter may reduce fermentation time, minimize dry matter losses, avoid contamination with pathogenic and toxigenic bacteria and molds and minimize the risk of incidental micro-flora causing off-flavor. Overall, this dissertation work is intended for the better utilization of sorghum by the parallel study of different fermentation states and starters.

1.5 Limitations of work

- Only two *murcha* samples were used.
- Only single kind of mold and yeast were isolated.
- Biochemical tests of isolated mold and yeast was not done for their confirmation.
- Optimization of fermentation parameters like temperature and pH could not be studied due to time and technical constraints.
- Shelf-life of the *Jand* could not be studied due to time constraints.
- Anti-nutritional factors and amino acid profile of the sorghum could not be studied.
- Protein digestibility of sorghum before and after fermentation could not be studied.

Part II

Literature review

2.1 Historical background of alcoholic beverages

Alcoholic beverages are believed to have originated in Egypt and Mesopotamia around 6000 years ago and it would appear that all civilizations and cultures have developed some form of alcoholic drink. The use of wheat, rye, millet, rice, oats, barley, potatoes or grapes in early fermentation processes paved the way to the technologies that are in existence currently (K. L. Jones, 1985).

Despite this early application of microbiology, the ability of microorganism to induce chemical changes was demonstrated several years later. Alcoholic fermentation was first identified in 1810, but at that time yeast was not identified as causative organism and later demonstrated that yeast could produce alcohol and carbon dioxide when introduced in sugar fungus, from which the name Saccharomyces originated (Prescott and Dunn, 1987).

The yeast cells growing under anaerobic conditions caused the conversion of glucose to alcohol and researchers also demonstrated that fermentation could be carried out using cell-free yeast juice, which led to the discovery of the role of enzymes in fermentation. He called the enzyme "Zymase" (Casida, 1997). Such work of pioneers finally revealed the truth that the alcoholic fermentation was in fact anaerobic, due to the presence of an enzyme complex known as Zymase, which is made available by the yeasts. Having realized the importance of yeasts in fermentation, people started culturing valuable yeasts and exploiting them for the production of various alcoholic beverages. Today, yeasts are utilized throughout the world for the production of alcoholic beverages in many different forms and tastes. The starting materials normally comprise either sugary materials, which need to be hydrolyzed to simple sugars before fermentation (Smith, 1996).

2.2 Raw materials for fermentation

A wide range of raw materials can be used for fermentation. The raw materials may include meat (for fermented sausages), milk (for yogurt, cheese, etc.), legumes (for *natto*, tempeh, *kinema*), cereals (for beer, whiskey, fermented porridge, etc.), fruit juices (for wine, brandy, vinegar), and vegetables (for lactic fermented products like sauerkraut,

pickles) (Pederson, 1971). A mixture of these substrates can also be used to develop newer types of fermented foods. For the preparation of starter cultures, rice, wheat, wheat bran, soybean and maize grits have been used (Hesseltine, 1965; Yadav, 1993).

2.3 Starchy raw materials for the production of alcoholic beverages

Starch, which has been gelatinized by heating, can be readily hydrolyzed to fermentable sugars by enzymes. Such starch occurs in cereal grains (rice, wheat, barley, millet etc.), root crops (cassava), or tubers (potatoes). All of these materials have been used for the production of whiskey, and the uses of potatoes for the production of vodka are well known (Prescott and Dunn, 1987). In Nepal, cereal grains (rice, wheat, barley, millet etc.) are used for the production of traditional alcoholic beverages viz. *jand* (undistilled) and *rakshi* (distilled) using *murcha* as a starter. Although the term *jand* is commonly used in the finger millet beer, beers from maize, rice etc. are also called *jand* the name of the beer is deriving from the raw material used for fermentation e.g. *makai jand* (maize beer), *vate jand* (rice beer) (Tamang *et al.*, 1988). Although various cereal grains are used for the production of *jand*, the one prepared from finger millet, for various reasons, will possess unmatchable quality.

2.4 Some alcoholic beverages from cereals

2.4.1 Alcoholic beverages prepared from millet

As cited by (Pederson, 1971), Thumba is an alcoholic beverage produced from millet in West Bengal. The millet seeds are boiled, cooled and inoculated with yeast and fermented for ten days in section of Bamboo. The yeast is generally sold as small cakes in the market places which has been identified as *Endomycopsis fibuligera.*

Chhang is the millet beer brewed in Sikkim (Pederson, 1971). Similarly *Braga* is a fermented drink prepared from millet in Romania (Pederson, 1971).

2.4.2 Maize

The early settlers in America met with many types of beverages prepared with corn as the main ingredients. Sir Walter Raleigh was introduced in 1587 to *Pogotowr* an Indian beer made from corn. The South American Indians used corn in their preparation called *Chica*

or *Chichara* a light beverage and Sora, a heavy beverage, were names applied by the Incas for corn beers. In addition, (Mabesa, 1986) reported a traditional Ugandan alcoholic beverage prepared from maize known as *Kweete.*

2.4.3 Rice

Japanese "Sake" is a clear, pale yellow, rice wine, with a characteristic aroma, little acid and slight sweetness (Murakami, 1972). The alcohol content may vary between 14-20 %(v/v) (Humphreys and Stewart, 1978).

It is generally believed that the technique originated in China, but comparison of the production processes for sake and Chinese alcoholic beverages shows marked differences, especially in respect to the microorganisms concerned. According to earliest records, sake was originally brewed from rice that has been chewed to achieve saccharification, followed by natural fermentation. Sake brewed in this way was used as a sacred wine in the worship of the Shinto gods (Humphreys and Stewart, 1978).

It has a flavor somewhat resembling sherry (Pederson, 1971). Its aroma is characteristic & owes much to the koji (saccharifying agent) used in its preparation. On the palate the beverage gives ample evidence of its alcohol content with no astringency, little acidity, & slight sweetness. In Japan it is often served warm, especially in winter (Humphreys and Stewart, 1978).

The starch of steamed rice is saccharified by the mold, *A. oryzae*. The koji thus produced is added to a thin paste of fresh boiled rice. Fermentation by the yeast, *S sake*, is then initiated & may continue for 30-40 days. More rice & koji may be added to continue the fermentation. It is finally filtered, pasteurized & bottled (Pederson, 1971).

2.4.4 Wheat

Traditionally, beer is made with malted barley but wheat beer substitutes a substantial proportion of wheat for the barley. This changes things greatly. The beer is lighter in mouth-feel. A wonderful acidity creeps into the brew, ensuring the sensation of freshness (Reed and Peppler, 1973).

A top fermented beer, called Weissbier, is brewed in Germany, particularly in Berlin area which is prepared from malted wheat rather than barley. Weissbier is sold with yeast

present in bottle (Helbert, 1987a). The popularity of this style is well deserved since not only does it taste great but it is a healthy drink because of its moderate alcohol and good vitamin B and trace mineral content (Woolfolk, 1971).

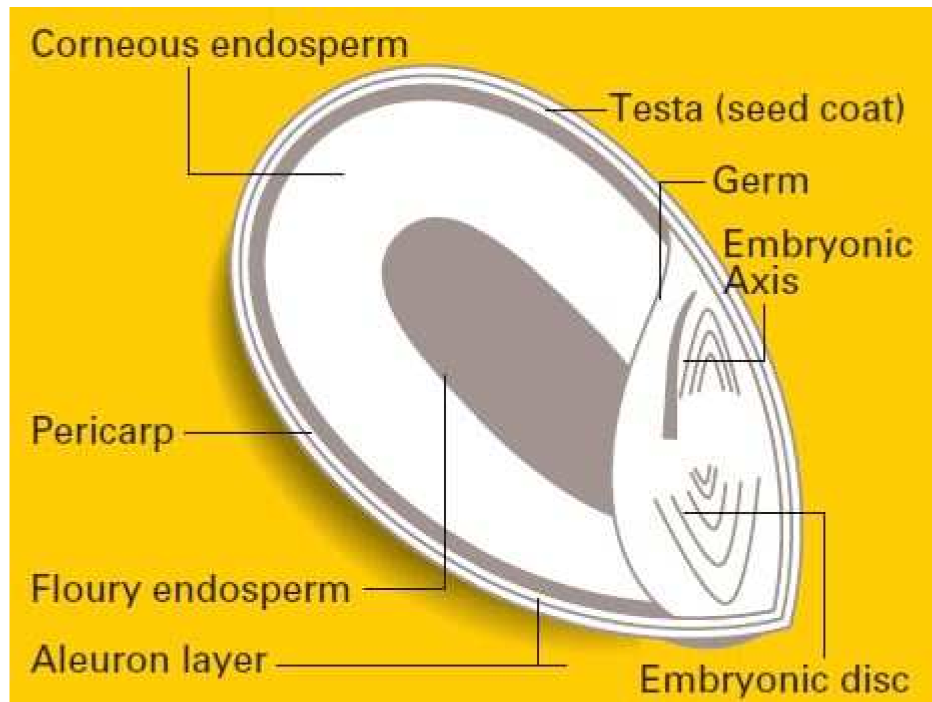
Another to fermented beer produced in the Continent is called Lambic beer, which is peculiar to the Brussels area. The mash is prepared from grist containing 60% malted barley and 40% raw wheat (Helbert, 1987a).

2.5 Sorghum

Sorghum (*Sorghum bicolor L. Moench*) is used extensively in some parts of Africa and Asia. Its world production reaches 70 million tones, in the early 1980's and it is the fourth most important cereal crop in the world after wheat, rice and maize (ISI, 2001). Sorghum is the most important cereal crop in Sudan, it occupies about 40-48% of the total area used for the main field crops, and average consumption is about 96% of the total production, which is about 3.31 million tones (FAO, 1996).

2.5.1 Anatomy of sorghum seed

Sorghum seed consists of 3 major anatomic sections - pericarp (outer layer), endosperm (storage organ) and the germ. The pericarp that is expressed from the ovary wall is made of 3 segments -epicarp, mesocarp and endocarp. The epicarp is the outermost layer and usually covered with a thin waxy film. The thickness of the mesocarp, the middle structure, varies from the very thin cellular remnant of small amount of starch granules to 3 or 4 cellular layers containing a large amount of starch granules. Sorghum is the only food grade crop that is reported to contain starch in this anatomical section. The endosperm is a storage organ that is comprised of aleurone layer, peripheral, corneous and floury areas. The aleurone contains proteins (protein bodies, enzymes), ash (phytin bodies) and oil (spherosomes). The germ is comprised of 2 major parts, the embryonic axis and embryonic disc. The protein of the germ contains high levels of lysine and tryptophan that are excellent in quality (Britannica, 2017). Fig. 2.1 shows the anatomic sections of sorghum seed.



Source:(Cleveland, 2013)

Fig 2.1 Anatomy of sorghum seed

2.5.2 Classification, distribution and adaptation of sorghum

Sorghum belongs to the family *Gramineae*, tribe *Andnopogoneae* and sub-tribe *Sorghastrae* (Onwveme and Sinha, 1991). *Sorghum bicolor L. Moench,* other common names for sorghum include great millet, guinea corn (West Africa); kafir corn (South Africa); milo, sorgo (United States); kaoliang (China), dura (Sudan); Mtama (East Africa), jola, jawa, cholam (India) (Purseglove, 1979). Millets and sorghum are cereal grains widely grown in Africa and elsewhere. They survive drought conditions better than maize and other cereals, and so are commonly grown in areas where rainfall is low or unpredictable (Latham, 1979).

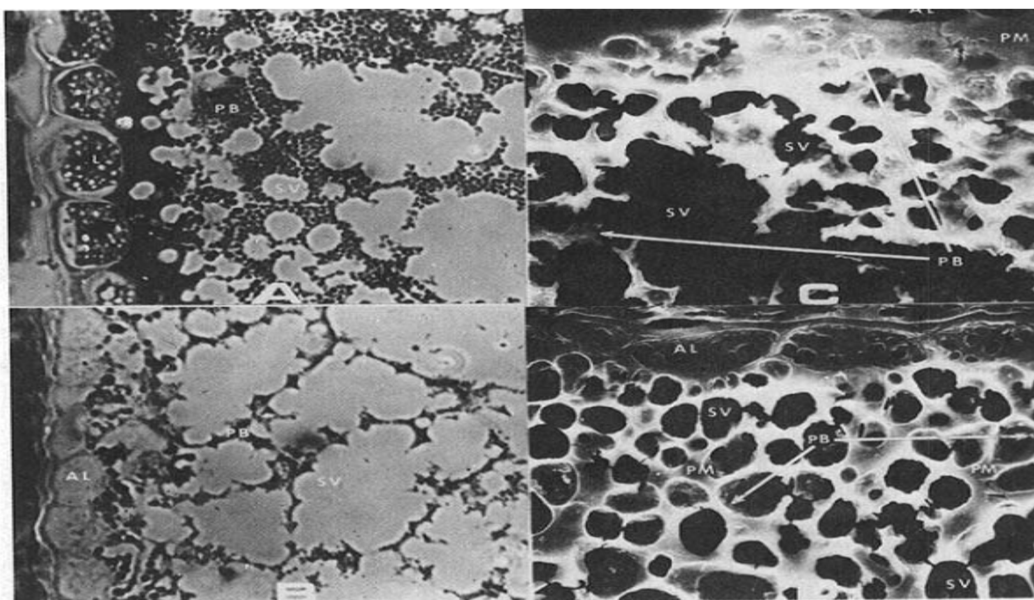
Grain sorghum is the cultivar grown principally for its grain, although some may be harvested for fodder or silage; grains relatively large palatable, threshing free from glumes; stalks usually moderately dry with little sweetness. They may be divided in USA into: Milo, Kafir, Hegari, Feterita and Hybrid grain sorghums (Purseglove, 1979). There are many varieties of sorghum, most of which grow tall and have a large inflorescence, there are also dwarf varieties. The grain is usually large but varies in colour and shape with type.

Sorghum requires more moisture than millet, but less than maize (Latham, 1979). Sorghum, common name for maize- like grasses native to Africa and Asia, where they have been cultivated since ancient times, up to 3 m (10 ft.) tall, they bear seeds on terminal heads or panicles. Grain sorghums are the staple food for millions of people in China, India and Africa; elsewhere. Sorghums serve primarily as livestock feed. Sorghum is grown in Africa (16%), Asia (36%), Central and South America (21%) and USA (20%), respectively (ISI, 2001).

2.5.3 Endosperm characteristics of sorghum grain

The endosperm of sorghum is composed chiefly of storage parenchyma cells filled with starch granules embedded in a continuous matrix of protein (Watson *et al.*, 1955). It contains 3 regions of both floury and horny endosperm. Starch is the major component of sorghum endosperm. According to the type of starch in their endosperms, the sorghum grains can be classified into waxy and non-waxy. Starch consists of amylose, a linear glucan with α-1, 4 linkages, and amylopectin, a highly branched glucan with α-1,4 and α-1,6 linkages. Non-waxy sorghum contains starch that is composed of approximately 25% amylose and 75% amylopectin, whereas starch in waxy sorghum is almost 100% amylopectin (Sikabbubba, 1989).

Non-waxy sorghum starch is highly resistant to enzymatic digestion, whereas waxy starch is highly susceptible to enzyme digestion (Sullins and Rooney, 1975). In non-waxy sorghum flour, there is a high concentration of protein bodies in the peripheral endosperm area, while the protein bodies are more evenly distributed in waxy sorghum endosperm (Fig. 2.2). The higher the content of amylose, the lower is the digestibility of starch (Aarathi *et al.*, 2003). Previous study have shown that there are apparent differences in the digestibility of amylose and amylopectin (Goddard *et al.*, 1984). The possible reason is that amylopectin has a larger surface area and is highly organized, leading to the formation insoluble aggregates (Aarathi *et al.*, 2003).



(a) Non-waxy Kafir (b) waxy Kafir

Source:(Buren and Robins, 1969)

Fig. 2.2 Light and scanning electron photomicrographs comparing the endosperm structure of non-waxy Kafir and waxy Kafir (Sullins and Rooney, 1975). Left, peripheral endosperm; right, central endosperm.

2.5.4 Utilization

Grain sorghum ranks third among cereals for human consumption, and is a staple food in Africa, China and India, superseded only by rice and wheat. It is the most important cereal crop in Sudan. Sorghum flour is utilized in Sudan in the form of a fermented product (ElTinay *et al.*, 1979). Some 30 important food products are made from sorghum in Sudan. These can be grouped into two, those made from ungerminated grain and those made from sorghum malt. The former are the more important, being the true staple that go with various relishes and sances, and the latter group forms the beverages and snacks (Dirar, 1993).

Sorghum is a major food crop in the semiarid tropics of Africa and Asia. In these areas where the people are typically poor and food resources are limited. Sorghum is the major source of protein, sorghum food products of high protein quantity and quality is essential. Traditionally in these developing countries, sorghum is consumed as porridge or flat bread (Kirleis, 1990). The sorghum plant has physiological adaptation mechanism that makes it

suitable for low rainfall areas. The opportunities for improving the commercial value of sorghum depend on the fact that sorghum can be used as food for humans and as feed for livestock.

To make sorghum popular, new product development needs to be undertaken and this includes revival of indigenous dishes (Madibela and Lekgari, 2005). There are opportunities to increase the product base made from sorghum. These include refined flour, different brands of beer, infant foods, bread flour, breakfast cereals and pet foods. Sorghum is a major source of protein and calories in the diets of a large segment of the populations in Africa and Asia. Its most important cereal crop in Sudan, it is a security crop widely grown and produce 18 million tons (FAO, 1997). But the nutritional quality of sorghum generally is poor and it is deficient in many nutrients such as lysine, essential amino acids, which makes sorghum protein inferior in quality (Eggum *et al.*, 1983).

2.5.5 Proximate composition of sorghum

2.5.5.1 Moisture content

Khattab *et al.* (1972) reported a range of 6.4 – 9.6% for sorghum moisture content; Yousif and Magboul (1972) reported a range of 5.7 – 10.7% for moisture content of different varieties of sorghum, while Marfo *et al.* (1990) found that the moisture content of different sorghum cultivars range from 6.0 – 14.0%. Shadad (1989) reported a range of 6.5 to 8.0% for moisture content of twenty sorghum varieties.

2.5.5.2 Ash content

Khattab *et al.* (1972) reported a range of 1.3 – 2.5% ash content for eight sorghum cultivars. Elmaky (1994) analyzed five cultivars and the range was 1.5 – 1.9% ash content. Osman (2005) reported an overall mean of 1.5% ash content for four sorghum lines.

2.5.5.3 Fat content

Marfo *et al.* (1990) stated that sorghum contains 5-6% fats. Yousif and Magboul (1972) found a range of 3.0 – 4.1% fat content for fifteen sorghum cultivars.

2.5.5.4 Fiber contents

Osman (2005) reported a mean of 1.6% fiber content for four lines of sorghum. Yousif and Magboul (1972) reported a fiber contents for fifteen sorghum cultivars ranged from 1.2 to 3.5%. Hulse *et al.* (1980) studied fiber content of different sorghum varieties and reported a range of 1.0 – 4.2%. A higher value for sorghum fiber content was reported by Dendy (1995) which was 6.6%.

2.5.5.5 Protein content

ElTinay *et al.* (1979) analyzed three local sorghum cultivars grown in Sudan and found protein content in the range of 6.8 to 8.8%. Yousif and Magboul (1972) studied protein content of fifteen different varieties of sorghum and gave a range of 6.9 to 12.8%. Bello *et al.* (1990) reported protein content of three sorghum cultivars to range from 13.2 to 14.3%.

2.5.6 Protein fractions

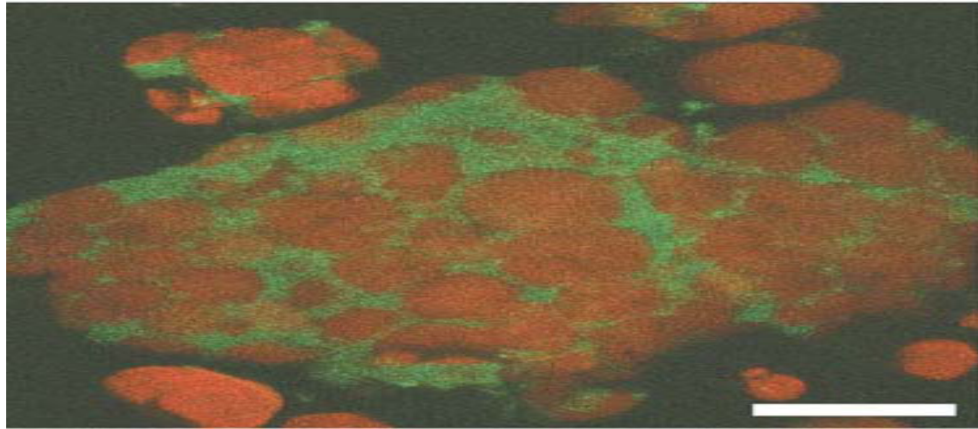
Corn protein can be separated into six fractions by selective extraction method: albumin, globulin, zain, G1-glutlin, G2-glutelin and G3-glutelin. Sorghum prolamin fraction has been divided into three sub fractions, α- β- and γ- kafirins (Shull *et al.*, 1991). Sorghum proteins are divided proteins into five major groups: albumins (soluble in water), globulins (soluble in salt), prolamins (soluble in 70-80% ethanol), glutelins (soluble in sodium hydroxide) and scleroproteins (insoluble in aqueous solvents).

The water and salt soluble fractions are comparatively superior in nutritional quality. Studies on amino acid composition of protein fraction showed that albumin and globulin fractions contained high amount of lysine and tryptophan and in general were well balanced in their essential amino acid composition, so the increase in the albumin fraction will result in improvement of the nutritive value of the grain (Wu and Wall, 1980).

On the other hand, the lysine which is the most deficient in sorghum protein present in much higher quantities in the glutelin fraction than in the prolamin fraction. Any change in protein composition which would increase the prolamin fraction will result in a decrease in the lysine content, and any increase in glutelin fraction will result in an increase in lysine level of seed (Virupaksha and Sastry, 1968).

Hamza (1999) reported these different values for protein fractions of whole sorghum seeds: Globulin plus albumin was 17.18%, Kafarin was 16.15%,Cross-linked kafarin was 25.40%, Glutelin-like was 6.91%, True glutelin was 28.61% while the non-extractable protein was 10.13%. Ibrahim *et al.* (2005) stated that the albumin plus globulin fraction was increased during the first 8h of fermentation while other fractions were observed to fluctuate during fermentation time. Yousif and Magboul (1972) studied the effect of fermentation on sorghum protein fractions and found that the globulin plus albumin fraction increased significantly during the first 8h of fermentation. Kaffirin fraction decreased during the first 8h of fermentation but increased sharply as fermentation process going on. Glutelin like protein, which was the minor fraction, true glutelins, and the second most abundant fraction, together with non-extractable proteins fluctuated during the fermentation process.

During development of sorghum seed, kafirins are synthesized and deposited inside the rough endoplasmic reticulum to form protein bodies. Within the protein body, the kafirins are distributed in a nonhomogeneous fashion. Immunocytochemistry showed that α-kafirin is located in light staining areas mainly in the interior of the protein body, and β- and γ-kafirin are found in dark staining areas inside and at the periphery of the protein body (Shull *et al.*, 1992). Protein has been found to line the channels that lead into the interior of sorghum granule (Han *et al.*, 2005). Those proteins could interfere with inward migration of α-amylase during digestion. It was suggested that γ-, and to a lesser extent β-kafirins, form a disulfide-bond enzyme-resistant layer at the periphery of protein bodies that restricts access by proteases to the easily digestible α-kafirin (Choi *et al.*, 2008). Fig. 2.3 shows laser scanning micrographs of sorghum floor showing protein and starch arrangement.



Source:(Choi *et al.*, 2008)

Fig. 2.3 Confocal laser scanning micrographs of sorghum flour, faint: protein, dark starch

2.5.7 Anti-nutrients in sorghum

Sorghum, one of the five leading cereal grains in the world, contains polyphenolic compounds that act as antioxidants and anticarcinogens. These compounds are important astringent, bitter and sour characteristics with its potential health benefits however, sorghum is worth exploring in value added food product (Brannan *et al.*, 1998).

Sorghum contain about 0.4 – 2.5% tannins (Rooney and Awika, 2005). Sorghum is unique among cereals because some cultivars produce polymeric polyphenol known as tannins (Butler, 1990). Tannins are often considered to be nutritionally undesirable, they form complexes with proteins, starch, minerals and digestive enzymes (Butler *et al.*, 1984; Salunkhe *et al.*, 1990). Tannins have been shown to reduce bioavailability of iron and vitamin B12 (Leiner, 1980). Also the proteinase inhibitors were earlier detected in sorghum especially trypsin and chymotrypsin inhibitors (Harishkumar *et al.*, 1978). Also tannins affect the growth of animals in three main ways, they have astringent taste, which effects palatability and decrease feed consumption, they form complexes with proteins and reduced digestibility, and they act as enzyme in activators (Buren and Robins, 1969).

Different processes has been reported to remove or inactivate tannins in sorghum, through direct removal, extraction, cooking, germination, fermentation, combination of germination and fermentation, and incubation with malt (Salunkhe *et al.*, 1990). Fermentation of sorghum commonbeen tempe-based weaning foods deceased phytate by

44% and increase the *in vitro* protein digestibility by 88% (Mogula, 1992). Phytic acid is myo-inositol, phosphorylated on all of its 6-hydroxy groups, it can bind ionically to proteins and interferes with minerals mainly Zinc. Phytates bind minerals in the gastrointestinal tract making dietary minerals unavailable for absorption and utilization by the body (Haug and Lantzch, 1983). Phytate level is found to decrease during certain food processing operation to various extent like milling, soaking, germination, fermentation and heat treatment (Mahgoub and Elhag, 1998).

2.6 Traditional alcoholic beverages of Nepal

In Nepal, the history of alcoholic beverage dates back to ancient times. These technologies were developed by ethnic groups while celebrating various festivals and settlement of marriage. The knowledge of home brewing has been passed on to generations but they are quite ignorant about the broad dimensions of microbial biochemistry or their complex mechanisms. In fact the exact nature of fermentation is still not fully known to them (Gajurel and Baidya, 1979).

Among the various fermented foods, *Jand* (*Chhyang* or *Toongba* or *Poko*) and *Rakshi* are the major alcoholic fermented liquor traditionally consumed in various parts of Nepal, depending on the availability of the raw materials. *Murcha* (yeast) starters are common for the necessary fermentation to produce these products in Nepal, some parts of India and southeast China (J. P. Tamang *et al.*, 1988).

2.6.1 Jand

Jand is a generic term that refers to Nepalese traditional sour-sweet cereal beer made from grains like millet, rice, wheat, etc., by using *murcha* as the starter culture (Subba *et al.*, 2005) and bears similarity with many traditional beers of the world (Dahal *et al.*, 2005). *Jand* is very popular among the rural mass of Nepal (Rai,1991). However, with the advent of alcoholic beverages based on 'new' technology, this product has earned itself social stigma among the urbanites: it is regarded as poor man's wine. This fact notwithstanding, the annual production of *jand* is higher than that of any other indigenous fermented products and this trade is probably the single-most important economic activity among most ethnic groups of low income category (Subba *et al.*, 2005). Some aspects of *jand*

have been reviewed by (Aidoo *et al.*, 2005). They have described the role of *mucaraceous* fungi in producing amylase needed to saccharify and liquefy starch.

The amylase activity has been reported to reaches its peak on the second day of fermentation. The authors have also mentioned the presence of mixture of yeasts (*Pichia anomala, Saccharomyces cerevisiae, Candida galbrata*) and lactic acid bacteria (*Pediococcus pentosaceus, Lactobacillus bifermentans*) in numbers exceeding 10^5 cfu/g in matured *jand*. *Jand* is served in different forms and modes. Strained *jand* is prepared by leaching out the readily extractable contents from the mash with luke warm water. The beverage is cloudy in appearance and has a very short shelf-life, of the order of few hours. The shelf life of strained *jand* can be extended to a few months by in-bottle heat treatment (pasteurization) but the time-temperature regime has to be worked out carefully to take into account the compounded influence of alcohol content, pH, acidity, total soluble solids, and packed volume of *jand* (Mongar and Rai, 2005).

The cereal of choice for *jand* preparation is finger millet (*Eleusine coracana Gaertn L*) but other cereals like maize, wheat, and rice are also used (Rai, 1991). Finger millet is often termed as poor man's cereal. In this light, *jand* preparation from this cereal can be viewed as an important value addition activity.

Table 2.1 Physicochemical properties of *jand* from different cereals

Parameters	Cereals			
	Wheat	Millet	Rice	Maize
pH	4.13	3.84	3.64	3.85
Total acidity (m/v) as % lactic acid	1.06	1.11	1.5	1.11
Alcohol as % (v/v)	8.38	7.15	7.37	8.07
Aldehyde (mg/L) as acetaldehyde	5.61	0.61	0.33	1.5
Reducing sugar (m/v) as % dextrose	15.84	7.04	22.88	22.88
Esters (mg/L) as ethyl acetate	8	6	7	5
Total soluble solids, °brix	1.75	0.48	2.61	0.38

Source: (Upadhyaya, 2005)

2.6.2 Nigar

Nigar is the clear liquid that spontaneously accumulates during prolonged anaerobic fermentation of *jand*. The product likens sake and is highly prized by the drinkers. *Nigar* can therefore be classified as cereal wine, rather than beer (Rai, 2006).

2.6.3 Rakshi

Raksi (also spelt rakshi, rukhsi) is an unaged congeneric spirit obtained by pot distillation of the slurry of *jand*. The product likens whiskey and has highly varying alcohol contents (KC *et al.*, 2004), generally between of 15 and 40% (Subba *et al.*, 2005). Several basic researches have been done on *raksi* production from different cereals using *murcha* starter as well as pure cultures isolated thereof (Bhandari, 1997; R. K. Rai, 1984; Shrestha, 1985; Subba, 1985; Yadav, 1993) but there seems to be general lack of attention towards process development such as preparation of good starter culture, increasing efficiency of traditional distillation apparatus, and separation of fients and foreshots for improving quality of raksi.

2.6.4 Hyaun thon

Hyaun thon is an alcoholic beverage (undistilled) indigenous to Nepal. The preparation methodology of *hyaun thon* is entirely different from other indigenous alcoholic beverage of Nepal. The unique features regarding its preparation are the blend of solid state and submerged fermentation and use of high inoculum in the form of *mana*. Due to submerged condition the growth of mold is questionable. The high inoculum acts as substrate, coloring agent and source of yeast and enzyme.

In solid state fermentation scale up is hard due to large surface area but *hyaun thon* preparation is submerged fermentation so there is no problem for the scale up so *hyaun thon* preparation has some commercial potential.

2.7 Fermentation starters for cereal fermentations

Cereal fermentation requires a saccharification process, which is accomplished with some difficulty. In the west, saccharification of cereals is achieved by using malt as a source of amylase. However, in Asia the malting process is rarely used in traditional fermentation processes. Instead, fermentation starters prepared from the growth of molds on raw or cooked cereals is more commonly practiced (Lee, 1999). The starters used for cereal fermentations are therefore amylolytic fermentation starters (Aidoo *et al.*, 2005). Fermentation starters are referred to as chu in Chinese, nuruk in Korean, koji in Japanese, ragi in Southeast Asian countries, and bakhar ranu or marchaar (murcha) in India (Batra and Millner, 1974).

Lately, there has been considerable interest in the starter cultures for cereal fermentations. Current food biotechnological research in developing countries seems largely limited to the identification of microorganisms for starter culture development (Ruskin, 1992). Identifying and providing a practical means of using appropriate starter cultures are advantageous due to the competitive role of microorganisms and their metabolites in preventing growth and metabolism of unwanted microorganisms. A strong starter may reduce fermentation time, minimize dry matter loss, avoid contamination with pathogenic and toxigenic bacteria and molds, and minimize the risk of incidental micro flora causing off-flavor.

According to Nout (1992) and Harlander (1992) optimization of starter cultures may be achieved by either conventional selection-or mutation, or by recombinant-DNA techniques to result in increased levels of safety. Relatively little is known of the contribution of micro flora to the formation of desired flavor notes during such fermentations. Genes for flavor and other beneficial enzymes that come from incidental micro flora may be incorporated into starter bacteria to facilitate more subtle and ancillary aspects of the fermentation along with primary events such as lactic acid production, thus preserving the distinctive nature of products made in different regions (Haard, 1999).

Although development of such gradually-evolved and stable fermentation starters will be an attractive proposition for use in small-scale fermentations under non-sterile conditions, this will not be the most appropriate in all cases. In the exercise of upgrading traditional food fermentation techniques, it would therefore be worthwhile to investigate the effect of inoculum enrichment on product characteristics and consumer acceptance (Ruskin, 1992).

A different tool to stabilize fermentations under non-sterile conditions is the use of multistrain dehydrated starters, which can be stored at ambient temperatures, enabling more flexibility. Such homemade starters are widely used in several Asian food fermentations. These starters are more homogenous and their dosage is convenient, but because they are manufactured under non-sterile conditions, some are heavily contaminated with spoilage organisms. This requires quality monitoring of the inoculum and of the fermentation process in which it is used (Nout, 1992).

Other examples of durable home-prepared starter materials used in Asian food fermentations are Indonesian ragi and Vietnamese men tablets (Hesseltine *et al.*, 1988). Depending on their specific purpose, these dehydrated tablets, prepared from fermented rice flour, contain mixed populations of yeasts, molds, and bacteria. Ragi tablets can be stored up to 6 months and constitute a convenient starter material for application in home and small-scale industrial fermentations of rice or cassava (Nout, 1992).

2.8 Mixed culture fermentation

Mixed-culture fermentations are those in which the inoculum always consists of two or more organisms. Mixed cultures can consist of known species to the exclusion of all others, or they may be composed of mixtures of unknown species. The mixed cultures may

be all of one microbial group - all bacteria - or they may consist of a mixture of organisms of fungi and bacteria or fungi and yeasts or other combinations in which the components are quite unrelated. All of these combinations are encountered in oriental food fermentations (Hesseltine, 1992).

Mixed cultures are the rule in nature; therefore, one would expect this condition to be the rule in fermented foods of relatively ancient origin. Soil, for example, is a mixed-organism environment with protozoa, bacteria, fungi, and algae growing in various numbers and kinds, depending on the nutrients available, the temperature, and the pH of the soil. Soil microorganisms relate to each other - some as parasites on others, some forming substances essential to others for growth, and some having no effect on each other (Hesseltine, 1992).

2.8.1 Advantages and disadvantages of mixed culture starters

2.8.1.1 Advantages

- Product yield may be higher.
- The growth rate may be higher. In a mixed culture one microorganism may produce needed growth factors or essential growth compounds such as carbon or nitrogen sources beneficial to a second microorganism.
- Mixed cultures are able to bring about multistep transformations that would be impossible for a single microorganism.
- In some mixed cultures a remarkably stable association of microorganisms may occur. Compounds made by a mixture of microorganisms often complement each other and work to the exclusion of unwanted microorganisms.
- Mixed cultures permit better utilization of the substrate. The substrate for fermented food is always a complex mixture of carbohydrates, proteins, and fats. Mixed cultures possess a wider range of enzymes and are able to attack a greater variety of compounds.
- Mixed cultures can be maintained indefinitely by unskilled people with a minimum of training. If the environmental conditions can be maintained (i.e., temperature, mass of

fermenting substrate, length of fermentation, and kind of substrate), it is easy to maintain a mixed culture inoculum indefinitely and to carry out repeated successful fermentations.

- Mixed-culture fermentations enable the utilization of cheap and impure substrates. In any practical fermentation the cheapest substrate is always used, and this will often be a mixture of several materials.
- Mixed cultures can provide necessary nutrients for optimal performance. The addition of a symbiotic species that supplies the growth factors is a definite advantage.

2.8.1.2 Disadvantages

- Scientific study of mixed cultures is difficult. Obviously, it is more difficult to study the fermentation if more than one microorganism is involved.
- Defining the product and the microorganisms employed becomes more involved in patent and regulatory procedures.
- Contamination of the fermentation is more difficult to detect and control.
- When two or three pure cultures are mixed together, it requires more time and space to produce several sets of inocula rather than just one.
- One of the worst problems in mixed-culture fermentation is the control of the optimum balance among the microorganisms involved. This can, however, be overcome if the behavior of the microorganisms is understood and this information is applied to their control.

2.9 Traditional starter culture used in the context of Nepal

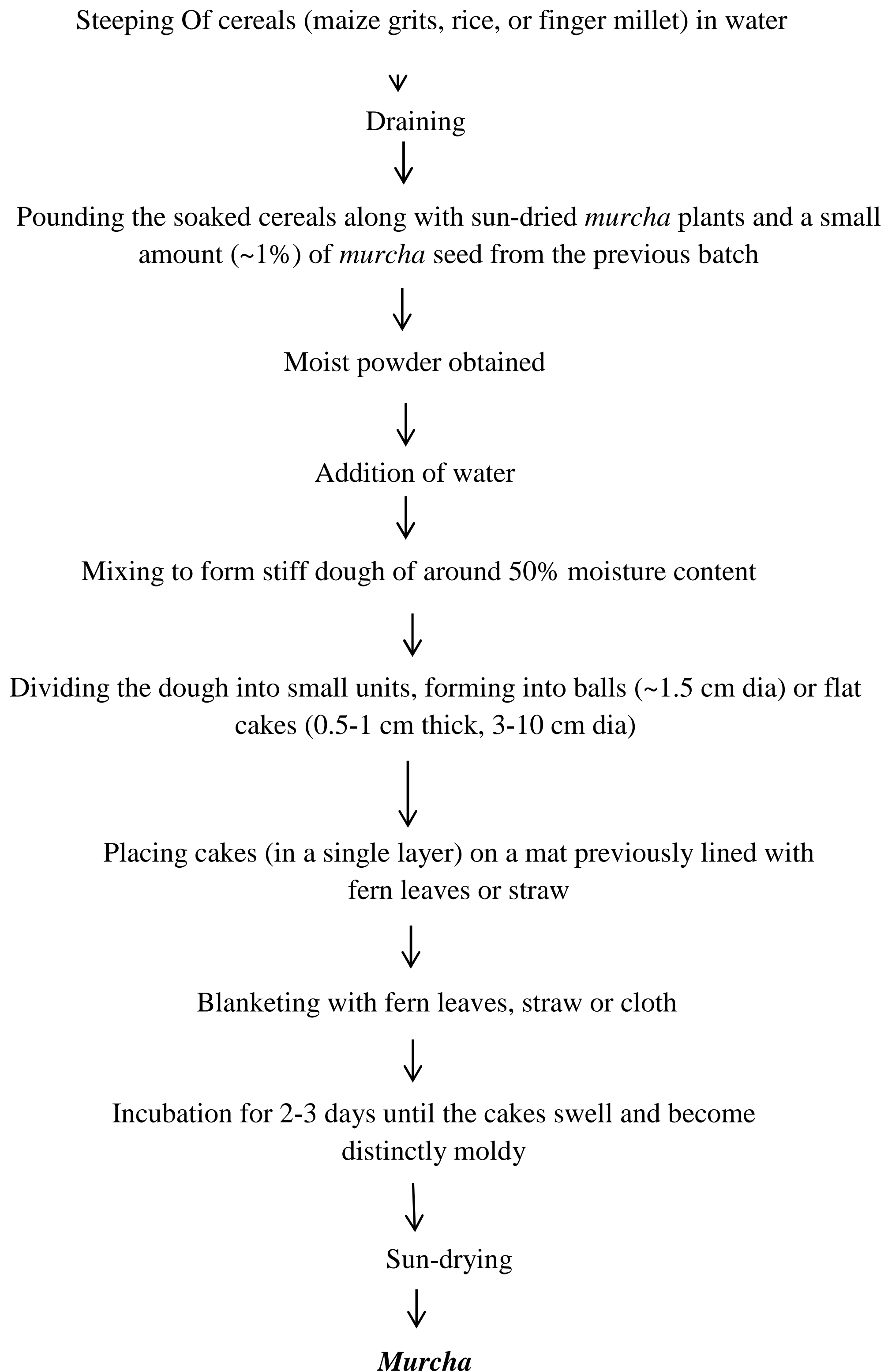
2.9.1 Murcha

Murcha (also spelt *marchaa, marcha*) is a traditional amylolytic starter used to produce sweet-sour alcoholic drinks, commonly called *jand* in the Himalayan regions of India, Nepal, Bhutan, and Tibet (China). Tsuyoshi *et al.* (2005) and Rai and Subba (2003) have described *murcha* as a starter cake employed as an inoculum in the production of traditional, cereal-based alcoholic beverages, viz., *jand* (undistilled) and *raksi* (distilled).

Murcha cakes are of two types, *manapu* and *mana* (Gajurel and Baidya, 1979). *Manapu* is prepared from rice flour and millet grains, whereas *mana* is prepared from wheat flakes or steamed rice.

Both the starter preparations are spontaneous fermentations in which the cultures come from the environment (straw). Since the microbial profile varies with the environment, season, and place, it stands to reason that the quality of *mana* can never be consistent.

The exact period of origin of these starters is not known. In Nepal, it is said that the customs of worshipping the god and goddesses were by Tantric process, and alcoholic beverages were offered during worship. This indicates that the existence of alcoholic beverage has a long tradition in Nepal. The traditional technology of *Mana* and *Mana*pu starter is kept so secret by some people of the Lubhu area in the Kathmandu Valley that is not even taught to daughters but only to daughter-in-laws (Gajurel and Baidya, 1979). It has become a tradition to prepare *Murcha* between September 10 and 20. There is a cultural belief that the quality of starter is better if it is made during this period because the day of Ganesh Chaturthi (a famous Hindu festival) generally falls within this period and *Murcha* starters prepared before this day are considered of good quality as cultural belief.



Source:(Subba *et al.*, 2005)

Fig. 2.4 General method of preparation processes of traditional *murcha*

2.10 Essential organisms for traditional cereal fermentation

In relation to the large number of microorganisms available, fermented foods and beverage utilize a limited number of microorganisms. They are broadly classified as fungi (molds and yeasts) and bacteria. Commercialized fermentations normally employ pure cultures but the traditional fermentations (and sometimes industrial fermentations as well) rely on starter cultures.

2.10.1 Yeasts

Yeasts are probably the oldest of microorganisms used (and cultivated) by man. Man has used them for bread making and alcoholic fermentation since prehistoric times. Today, yeasts are no longer limited to traditional uses, they find much more diverse uses than that could be thought of a century ago. Traditional uses apart, yeasts are now being increasingly used in genetic engineering, single cell protein (SCP) and enzyme production, vitamin production, microbiological assays, and flavor production (KC *et al.*, 2004).

Although yeasts are ubiquitous in nature only relatively small numbers of yeasts are used in the production of fermented and microbial foods. Some of those that enjoy a special status in food and fermentation industries are species of *Saccharomyces, Schizosaccharomyces, Candida*, etc. (KC *et al.*, 2004). All of today's 'culture yeasts' (commercial yeast cultures) are highly improved strains.

2.10.1.1 Yeasts in Traditional fermentation

The microbiology of the Nepali m*urcha* starters was analyzed for first time in the early 1990s. Nine samples of m*urcha* collected from Nepal were analyzed, and the result showed they all had a similar population of bacteria, yeasts, and molds (Tamang *et al.*, 1988). The yeast population in these samples was very high ranging from $5.4. \times 10^6$ to 6.1×10^8 cfu/g and mold count was 1.5×10^6 to 2.8×10^8 cfu/g. The identified yeasts were *Saccharomycopsis fibuligera*, *Pichia anomala*, and *Saccharomyces spp*.

The *Murcha* samples were also collected more recently in year 2000 from Nepal and the microbiological study carried out .Yeast and lactics were present in high numbers in m*anapu* starters, whereas molds were dominant in wheat-based m*ana* samples. In general, m*anapu* starters based on rice and millet showed a predominance of yeasts and

lactics in the range of 5×10^5 to 1×10^9 cfu/g. In general, m*anapu* starters based on rice and millet showed a predominance of yeasts and lactics in the range of 5×10^5 to 1×10^9 cfu/g. The m*ana* starters contained molds as dominant flora recording more than 1×10^7 cfu/g. Forty-five strains of yeasts, 29 strains of lactic acid bacteria, and 21 cultures of fungi were isolated and purified from various m*urcha* samples. Among the yeast, *Saccharomyces cerevisiae* strains were found to be dominant followed by *Candida versatilis* (H. N. Shrestha and Rati, 2002). *Endomycopsis*, one of the few types of yeast capable of producing amylases and using starch was also isolated from Lao Chao (a Poko like product also known as Chiu-niang or Tien-chiuniang by Chinese) (Campbell-Platt, 1994).

2.10.2 Molds

Industrially, molds are used in the production of fermented foods, enzymes, metabolites, antibiotics, and toxins. The industrially important genera of molds are found in the fungal subdivision *Deuteromycotina* (represented by *Penicillium* and *Aspergillus* species) and *Zygomycotina* (represented by *Rhizopus* and *Mucor* species). Molds are highly aerobic organisms and most of them grow best at an acidic pH and at a temperature of around 25°C.

Molds play a very important role in oriental food fermentations. They are used in the production of food and beverages ranging anything from *tempeh*, *sake t*o *jand*. In sake and *jand*, the molds are responsible for saccharifying the starch into simple sugars so that the latter can be utilized by yeasts for alcohol production. Some examples of notable amylolytic (starch hydrolyzing) molds are strains of *Aspergillus oryzae*, and species of *Mucor* and *Rhizopus* (KC *et al.*, 2004).

2.10.2.1 Mold in traditional fermentation

The microbiology of the Nepali m*urcha* starters was analyzed for first time in the early 1990s. Nine samples of m*urcha* collected from Nepal were analyzed, and the result showed they all had a similar population of bacteria, yeasts, and molds. The molds were *Rhizopus* and *Mucor*, all members of the *Mucorales* (J. P. Tamang *et al.*, 1988). Molds were dominant in wheat-based m*ana* samples. The m*ana* starters contained molds as dominant flora recording more than 1×10^7 cfu/g (H. N. Shrestha and Rati, 2002). *Mucoraceous*

molds, including *Rhizopus oryzae*, *R. chinensis*, and *Chlamydomucor oryzae*, have been consistently isolated from Lao Chao (a Poko like product also known as Chiu-niang or Tien-chiuniang by Chinese) (Campbell-Platt, 1994).

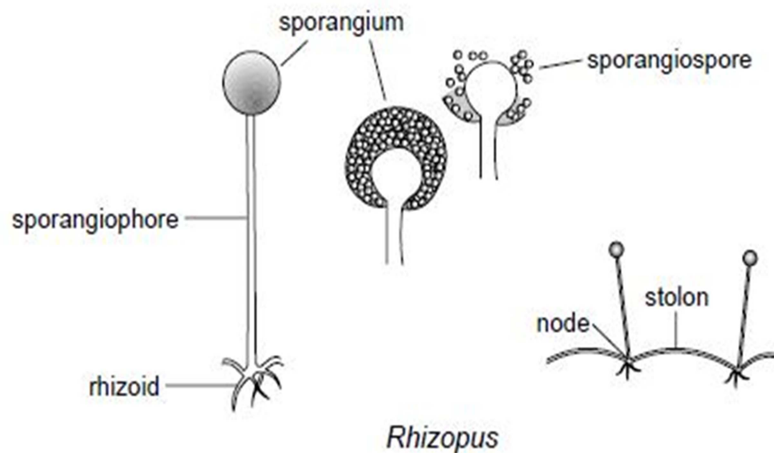


Fig. 2.5 Line diagram of *Rhizopus*

2.10.2.2 Saccharification of Cereals by mold

Unlike fruit and milk fermentations, cereal fermentation requires a saccharification process, which is accomplished with some difficulty. One primitive method of cereal saccharification would be chewing raw cereals and spitting them into a vessel in order to allow saccharification to occur through the action of salivary amylase, followed by alcoholic fermentation by natural yeasts.

Another method of cereal saccharification is through the malting process. Malting occurs naturally through wet damage of cereals during storage, and is used for beer making in Europe. However, in Asia the malting process is rarely used in traditional fermentation processes. Instead, fermentation starters prepared from the growth of molds on raw or cooked cereals is more commonly practiced. Fermentation starters are referred to as *chu* in Chinese, *nuruk* in Korean, *koji* in Japanese, *ragi* in Southeast Asian countries and *bakhar ranu* or *marchaar* (*murcha*) in Nepal & India (Batra and Millner, 1974).

The objective of saccharification is to convert starch to D-glucose as much as possible. Using glucoamylase it is possible to convert starch almost totally (99%) to D-glucose, but economically it is not feasible. Several important technical and economic variables interact to limit conditions allowing a maximum conversion to about 93-96% D-glucose, when

cornstarch is converted with glucoamylase. The kinetics of the saccharification of liquefied starch by glucoamylase is complicated because at any given time in the hydrolysis, a wide array of linear and branched dextrin are present causing many simultaneous reaction each with a different rate. The system as a whole has defined rigorous kinetic description. The amylose and amylopectin portion of cereal starch are converted by α-amylase during liquefaction to a collection of linear and branched dextrin. The linear dextrins are almost rapidly converted to D-glucose by glucoamylase. The branched dextrins are much less susceptible to hydrolysis owing to the lower rate at which glucoamyalse cleavage the α-(1, 6)-D-glucosidic linkage as compared to cleavage of the α-(1,4)-D-glucosidic linkage. For practical purpose, the dextrin hydrolysis reactions are irreversible. However, the hydrolysis to D-glucose is not complete because simultaneously condensation reaction occurs whereas D-glucose is condensed to reversion products.

The maximum quantity of D-glucose may be increased by treating starch with disbranching enzymes such as isoamylase and pullulanase to reduce the number of α-(1,6)-D-glucosidic linkage that impede rapid hydrolysis of starch by glucoamylase. It is necessary to conduct the hydrolysis at pH 5.9-6.3. Although glucoamylase (from *Aspergillus niger*) action is optimal at pH 4.3. The higher pH is necessary because of poor activity and stability of this type of pullulanase at lower pH values (Shrestha, 1985).

2.11 Biochemistry of alcohol fermentation by yeast

The organism uses EMP pathway, generating 2 ATP per mole of glucose converted to ethanol, plus CO_2. Ethanol, which is the end product, is primary metabolite. In an industrial fermentation, the basic strategy is to maintain Crabtree effect during the fermentation. A truncated form of the metabolic pathway for ethanol synthesis is given in Fig 2.6 below.

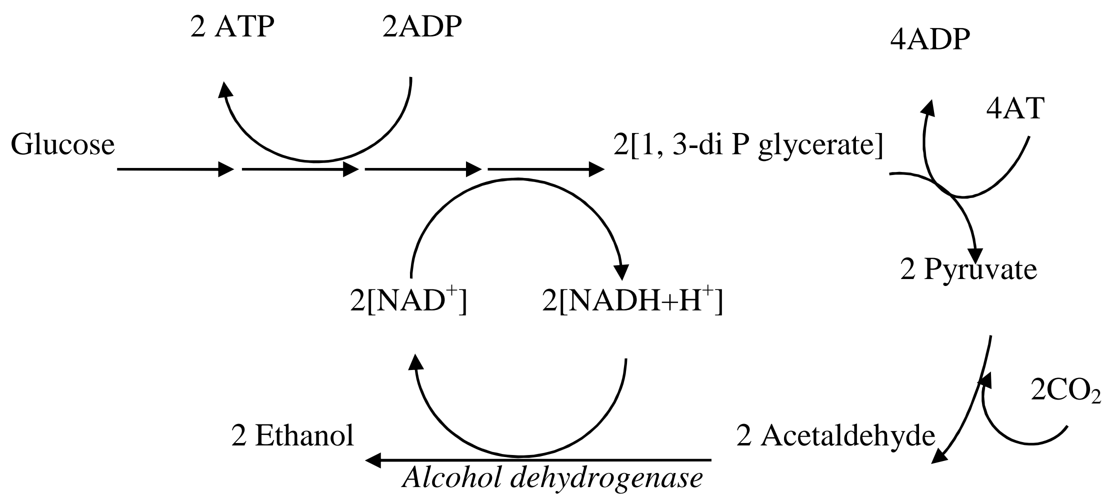


Fig 2.6 Simplified pathway of alcohol synthesis by yeast

2.12 Yeast isolation and identification

Methods for isolating yeasts from natural sources have been described by different authors (Barnett *et al.*, 1990; Hayford and Jespersen, 1998; Kaufmann, 1998; Kirsop, 1987), the protocols depending largely on the purpose and objective. Fermentative yeasts are cultured, for the most part, in Wickersham medium (Prescott and Dunn, 1987), commonly called Malt Yeast extract Glucose Peptone (MYGP) medium. Inclusion of malt promotes faster yeast growth and hence better isolation. The pH of the medium is kept at around 4.0 so that the condition becomes selective for yeast growth.

For the screening of fermentative yeast types that generally occur in starter cultures used in alcoholic fermentations, the method described by (KC *et al.*, 1999) seems rather straightforward. The method entails three stages, viz., (1) Enrichment (pre-fermentation) of the flora in sterile, 2% (w/v) high-test cane molasses for a week at 30°C (with occasional shaking for aeration), (2) Microscopic examination (negative staining) for the evidence of yeasts, and (3) Isolation by spread plating on MYGP agar.

Selective isolation of wild yeast types (for differentiation from culture yeasts) have also been described by different investigators (Helbert, 1987b; Kuhle and Jespersen, 1998).

Yeast identification methods range from traditional to very sophisticated ones. Traditionally, yeasts are identified by morphological and physiological criteria , but these

methods are very tedious (Granchi *et al.*, 1999). Auxanography (Woolfolk, 1971) and additional tests such as urea hydrolysis and nitrate assimilation are used for identification of species. Based on this principle, several rapid test kits for the identification of yeasts are also available (Umabala *et al.*, 2002).

Subba *et al.* (2005) have described a modification of the classical auxanographic technique. They claim that the method is much faster and economical than the conventional test and is particularly suitable when dealing with a small number of yeast types (less than 8). The modification of the routine method described here entails preparation of sugar plates on to which yeast isolates are inoculated. The response of yeast cells to different sugars are consistently and quickly established by this method. Modern methods of yeast identification (at the species level) are more sophisticated in that they utilize molecular techniques and have been described by several investigators (Granchi *et al.*, 1999; Hayford and Jespersen, 1998; Vasdinyei and Deak, 2003). Yeast genera are generally differentiated on the basis of mode of reproduction (vegetative or sexual), absence or presence of spores, and morphological characteristics of buds, spores, and yeast cell. Most methods use dichotomous keys for the differentiation of yeast genera.

2.13 Yeast cultivation, preservation and maintenance

A good review on yeast preservation and maintenance can be found in (Kirsop, 1987). Sub culturing (Active transfer) is the simplest method (although not very reliable) of yeast culture maintenance. Yeast isolates from plant sources can be maintained by active transfer (sub culturing every 3-6 months) and storage at refrigeration temperature without any significant change in functional properties (Rai and Subba, 2003). The cultural conditions adopted for the cultivation of yeasts depends on the type of fermented foods to be produced. The pH is generally maintained at 3.5-5.0. Although the optimum growth temperature of yeast is around 28°C, this temperature is seldom used for all types of yeast fermentations (except bakers' yeast and ethanol production). Beer or wine production in fact requires a temperature which is much lower than that needed for yeast proliferation. This is an example of how cultural conditions can be manipulated in order to produce specific fermented products.

2.14 Mold isolation and identification

Malloch (1997) has given an excellent account of mold isolation techniques. These techniques can be divided into two broad categories: (1) Direct methods, and (2) Selective methods. Both are routinely used in mycology laboratories and can be further divided into a number of subtypes.

For mold isolations from food samples, it is often most convenient to use the direct plating method, which involves direct inoculation of the material on nutrient agar amended with Martin's Rose Bengal Medium and then incubation for a few days (Malloch, 1997).

A very simple method for the isolation of saccharifying molds from *murcha* has been described by KC *et al.* (1999). The method entails spread-plating of broth sample on MYPG, and incubation at 30°C for 2-3 days to get mold colonies. The saccharification ability is tested by transferring the isolate on to cooked rice substrate and incubation for some days for the evidence of liquefaction.

Identification of molds is based almost entirely on the spore-bearing structures and on the spores themselves. The most common means of identifying molds is by the use of dichotomous keys, a system presenting a series of alternatives for consideration. The text keys are often accompanied by picture keys. Keys to sixty common genera of molds prepared by Malloch (1997) appear in Appendix E. The original version published in the internet is fully interactive, and provides faster identification. (Harrigan and McCance, 1976) have also given an excellent account of mold identification procedures.

Mold identification requires preparation of slides for microscopic examination. Some basic techniques have been described by several authors, including Aneja (1996), Malloch (1997), and KC *et al.* (2004), the last one being probably the simplest method. This method, termed "tape culture", basically involves inserting a cello-tape over the colony to stick the 22 spores and mycelia followed by adhesion on a clean slide for microscopic examination under objectives of different magnifications.

2.15 Cultivation, preservation and maintenance of molds

Molds can be cultivated in the laboratory in solid as well liquid media, which in turn can be semi synthetic, synthetic, or natural. Some of the common media used in mold

cultivation have been discussed by Malloch (1997). The choice of the medium of course depends on the type of mold to be isolated. Asepsis is critical in all aspects of cultivation of molds. For food fermentations, Potato dextrose Agar (PDA) and Malt Yeast extract Peptone Glucose Agar (MYPGA) are satisfactory.

When industrial cultivation of mold is required, for metabolite and food production in particular, solid-state fermentation is carried out in substrates such as rice (Wang and Hesseltine, 1987), wheat bran (Boing, 1987), etc.

The culture can by managed by many methods, such as in soil or in slants. In most traditional fermentations, the cultures are maintained as starter cultures such as *ragi*, *koji*, and *murcha* (Lee, 1999).

2.16 Bacteria

A number of bacteria find use in fermentation industries. In indigenous food fermentations, two groups of bacteria are extensively used, viz., (i) Lactic acid bacteria (LAB) in fermented dairy products and fermented vegetables, and (ii) Proteolytic bacteria, mainly of the genus *Bacillus* in alkaline fermentations. Sometimes, a mixture of these organisms can be used to prepare special products (Pederson, 1971)

2.16.1 Lactic acid bacteria (LAB)

Lactic acid bacteria are a group of Gram-positive, non-spore forming rods or cocci and most are aero tolerant anaerobes. They ferment carbohydrates to lactic acid as the principle component. Lactic acid bacteria are extensively used in the production fermented milk products, fermented vegetables, pickles, and fermented meat products. They also occur in almost all of the cereal-based traditional alcoholic fermentation. This is advantageous because the acid produced by the bacteria makes the medium favorable for yeast metabolism needed for alcohol generation. The acid also provides better sensory quality to the beverage (Rai, 2006). The LAB that is almost always involved in traditional alcoholic fermentations are species of *Pediococcus*, *Pediococcus pentosaceous* in particular (Aidoo *et al.*, 2005). Recently, *Pediococci* found in fermented foods have been shown by various workers (Guerra and Pastrana, 2002; Horn *et al.*, 1998; Wu *et al.*, 2004)to produce pediocin (an antibiotic) that is inhibitory to pathogens, *Listeria* in particular organisms.

2.16.2 Proteolytic bacteria

The single most important genus in this group is *Bacillus*. This genus is represented by Gram-positive, endospore forming rods. *Bacillus cereus* is a pathogen, implicated for occasional food-borne illness (Adams and Moss, 1996) whereas *Bacillus subtilis* is an organism of great significance in oriental soybean fermentations such as *natto* (Wang and Hesseltine, 1987) and *kinema* (T. B. Karki *et al.*, 2005).

2.17 Alcoholic fermentation

Alcoholic fermentation is simply the production of alcohol by using carbon and nitrogen substrate (Kausik and Yadav, 1997). Sugar and nitrogen compounds are the principal substrates for alcohol fermentation (Prescott and Dunn, 1987).

It is clear that cereal fermentation resulting from use of *murcha* is simply the result of concerted action of molds, yeasts and bacteria on the cooked substrate. The generalized scheme of the actions of *murcha* flora on the cooked substrate (Subba *et al.*, 2005) is given in Fig. 2.7.

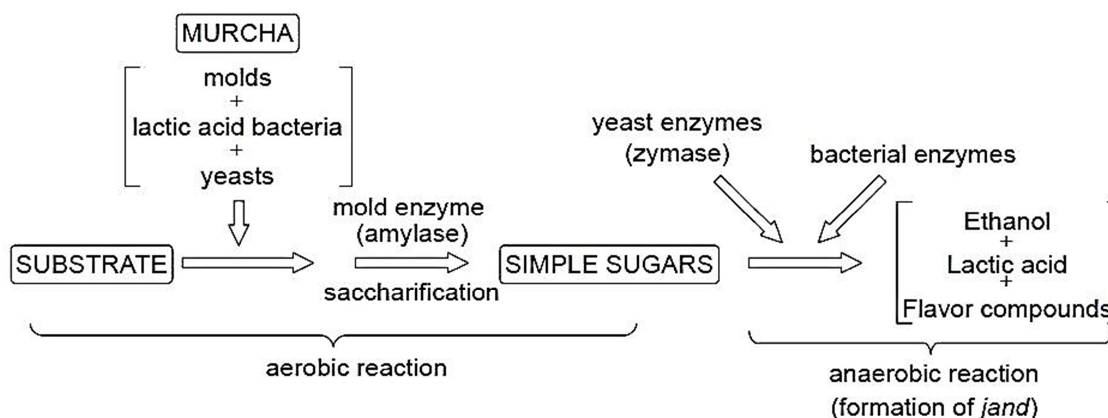


Fig. 2.7 Sequential and concerted action of *murcha* flora on cereal substrate

2.17.1 Stoichiometry

Ethyl alcohol is the product obtained from alcoholic fermentation of sugar by the action of enzyme *zymase* in yeast. In alcoholic fermentation one molecule of glucose produce two molecules of ethyl alcohol and carbon dioxide.

$$C_6H_{12}O_6 \xrightarrow{Zymase} 2C_2H_5OH + 2CO_2 + \text{Energy}$$

2.18 Flavoring compounds produced in alcoholic beverages

We do not usually drink alcohol as pure diluted ethanol, but as different alcoholic beverages. With improved analytical techniques, hundreds of different compounds have been identified in alcoholic beverages. The compositions and concentrations of these additional substances, congeners, differ very much from one beverage to another, and they determine the aroma of the drink. The organoleptic compounds produced by yeast except alcohols are: esters, aldehyde, organic acids etc. (Berry and Chamberian, 1986).

2.18.1 Esters

Esters are numerically the largest group of organoleptic compounds in alcoholic beverages. Lower esters have pleasant odors that are usually described as fruity (Barnett and Payne, 1990). Although some ester formation may occur during the distillation of spirits, the most common esters are produced by the yeast during fermentation stage. Nout (1992) proposed that esters were produced in the yeast cell by an enzymatic reaction between acetyl CoA derivatives of fatty acids and free alcohols rather than by an extra cellular chemical reaction. Experiments involving the addition of individual acids and alcohols have indicated that the there is competition between different alcohols and acids in ester formation such that the most abundant esters are derived from the most abundant acids and alcohols. Since ethanol is the most abundant alcohol the ethyl esters are the most abundant, followed by isoamyl and propyl esters. Acetate is the most abundant acid formed by yeast during fermentation, so acetate esters of ethanol and higher alcohols are the most abundant. Ethyl acetate at up to 50mg/l in beer and 175 mg/l in certain whiskies is the most abundant ester in alcoholic beverages (Batra and Millner, 1974). It has a characteristic fruity odor (Barnett and Payne, 1990).

2.18.2 Aldehydes

Aldehydes are synthesized by yeast as intermediates in the formation of alcohols through the decarboxylation of keto acids. The majorities are further reduced by alcohol dehydrogenase, but a small amount may be oxidized to acids. During the active phase of fermentation, excess quantities can be excreted into the fermentation broth. The corresponding aldehydes to most of the alcohols formed by yeast have been detected in

alcoholic fermentation (Engan, 1981). Acetaldehyde is thus quantitatively the most significant compound of this group as ethanol is the dominant alcohol formed during alcoholic fermentation. Generally, aldehydes have flavor threshold two to three orders of magnitude below the alcohols. The aroma of the lower aldehyde is generally perceived as fruity. Acetaldehyde has a characteristic pungent odor, but its solution in water, have an agreeable fruity odor (Batra and Millner, 1974). However, as the chain length increases they become more unpleasant, being cardboard-like and bitter (Messens and Vuyst, 2002).

Parameters which increases the initial fermentation rate, such as aeration, readily utilizable sugars and other nutrients, higher temperature, fast fermenting yeast strains and higher pitching rates result in increased accumulation of aldehydes (Greiger and Piendl, 1976).

The final concentrations of aldehyde in yeast fermentation are a balance between those which are formed in the initial stages of fermentation, and those which are utilized in the later stages. In addition, the presence of antioxidants which form complexes with aldehydes, such as sulphite ions and sulphur dioxide, can enhance the final concentrations (MacDonald *et al.*, 1984).

2.18.3 Organic acids

Some 100 organic acids have been reported in alcoholic beverages. These arrive from three areas of yeast metabolism. Those such as acetate, succinate, α-ketoglutarate, malate and citrate are derived from pyruvate via limited tricarboxalate acid cycle. Pyruvate itself constitutes a qualitatively important group of acids. They may have direct effect on flavor (e.g. the mouth feel flavor of pyruvate), but they also contribute to the pH of the beer. Some such as isobutyric and isovaleric acids are probably derived from the amino acid biosynthetic pathways, but the major groups are produced form malonyl CoA by the fatty acid synthetase pathway (Lynen, 1967). Shorter chain fatty acids such as hexanoic (caproic) acid, octanoic (caprylic) acid and decanoic (capric) acid are produced. They have been considered to have been leaked form the main biosynthetic pathway. These fatty acids are important flavor compounds in their own right and have been reported to give a caprylic, gouty, soapy or fatty flavor to beer and when released by autolysis during the maturation of beer they have been associated with a yeasty flavor (MacDonald *et al.*, 1984).

The acids present may be volatile of fixed. The term, volatile acid is rather loose one. It refers to the volatile fatty acid with steam. Besides acetic acid and lactic acid which is the normal by-product of alcoholic fermentation; formic, butyric, propionic and traces of other fatty acids are present. Acetic acid is not only a by-product of alcoholic fermentation but during the course of fermentation an appreciable amount may be utilized by the yeast. The volatile acids are produced mainly during the initial stage of alcoholic fermentation. More is formed in presence of oxygen than its absence (Amerine *et al.*, 1967).

2.18.3.1 Lactic Acid Production Using Lactic Acid Bacteria

Lactic acid (2-hydroxypropanoic acid) is an invaluable chemical. It was first discovered by the Swedish chemist Scheele in 1780, who isolated the lactic acid from sour milk. It was first produced commercially by Charles E. Avery at Littleton, Massachusetts, USA in 1881. Lactic acid can be produced by either microbial fermentation or chemical synthesis, a great deal of interest has recently become focused on the microbial fermentation, because the chemical synthesis of lactic acid is associated with several serious problems, including environmental issues and the depletion of petrochemical resources (Wee *et al.*, 2004). Lactic acid is classified as GRAS (generally recognized as safe) for use as a food additive by the US FDA (Food and Drug Administration) and it has been utilized in a broad range of applications in the food, beverage, cosmetic, medical and pharmaceutical industries (Naveena *et al.*, 2004) . Major use of lactic acid (accounts to 85% of demand) is still in food and food related applications.

Lactic acid is widely used in almost every segment of the food industry, where it serves in a wide range of functions, such as flavoring, pH regulation, improved microbial quality and mineral fortification. Moreover, lactic acid is used commercially in the processed meat, hams, fish and poultry industries, to provide products with an increased shelf life, enhanced flavor, and better control of food-borne pathogens. Due to the mild acidic taste of lactic acid, it is also used as an acidulant in salads and dressings, baked goods, pickled vegetables, and beverages.

Lactic acid is used in confectionery, not only for flavor, but also to bring the pH of the cooked mix to the correct point for setting. The advantages of adding lactic acid in confectionery include its low inversion rate, ease of handling, and ability to produce clear candies. Another potential application of lactic acid in the food industry is the mineral

fortification of food products. Lactic acid plays a vital role in the chemical industry, where it is used as a precursor for the syntheses of ethyl lactate, propylene oxide, propylene glycol, acrylic acid, 2, 3-pentanedione and dilactide. Another very promising lactic acid application is the production of environmentally friendly “green” solvents (lactate esters). They can replace traditional solvents made from petrochemical feedstocks (Tsai *et al.*, 1999).

Lactic acid bacteria have the property of producing lactic acid from sugars by a process called fermentation. Lactic acid bacteria genera include *Lactobacillus, Leuconostoc, Pediococcus, Streptococcus, Aerococcus, Carnobacterium, Enterococcus, Lactococcus, Vagococcus, Oenococcus* and *Weissella*. Lactic acid bacteria (LAB) can be classified into two groups: homofermentative and heterofermentative. The homofermentative LAB are *Lactobacillus delbrueckii, Lactobacillus plantarum, Lactobacillus bulgaricus, Lactobacillus helveticus, Lactobacillus casei, Streptococcus lactis, Streptococcus cremoris, Streptococcus faecalis, Streptococcus thermophilus* and *Pediococcus cerevisiae*. The heterofermentative LAB are *Leuconostoc mesenteroides, Lactobacillus cremoris, Lactobacillus brevis* and *Lactobacillus fermentum.*

The biological production of lactic acid *via* microbial fermentation has been studied extensively by a many research group (Hofvendahl and Hahn-Hagerdal, 2000). While the homofermentative LAB convert glucose almost exclusively into lactic acid, the heterofermentative LAB catabolize glucose into ethanol and CO_2 as well as lactic acid. The homofermentative LAB usually metabolize glucose *via* the Embden-Meyerhof pathway (*i.e.* glycolysis). Since glycolysis results only in lactic acid as a major end- product of glucose metabolism, two lactic acid molecules are produced from each molecule of glucose with a yield of more than 0.90 g/g. Only the homofermentative LAB are used for the commercial production of lactic acid (Yun *et al.*, 2003).

2.19 Production of toxic compounds

In addition to the production of carbon dioxide and ethanol during fermentation, microorganism also impart production of toxic compounds mainly methanol, aldehydes, higher alcohols etc, along with some organoleptic compounds which are important for flavor and bouquet of beverages (Reed and Peppler, 1973). Alcoholic fermentation of fruits and grains with yeast, usually *Saccharomyces cerevisae*, yields ethanol and very small

amount of other organic compounds. Occasionally methanol will contaminate the final product unless it is carefully removed by distillation. This methanol arises by demethylation of pectin by pectin esterase enzyme (Boing, 1987). Yeast do not form an enzyme capable of hydrolyzing pectin and consequently the reaction does not commonly occur in cereal fermentation. But pectin esterase is abundant in fungi. The ability of enzyme rises as the pH increases from 1 to 6 and the production of methanol goes up. If the grain with relatively high pH becomes contaminated with mold, the amount of methanol formed may be fairly high (Murakami, 1972).

According to Pilnik et al., 1981, fungi of species *Penicillium, Fusarium, Rhizopus* and *Seperotina* etc. contain pectin esterase enzyme. Similarly, bacteria of species *Clostridium* also contain this enzyme. Pectin esterase has a high specificity for the methyl esters of polygalacturonic acid. Pectin esterases have been described to hydrolyze ethyl, propyl and esters of polygalacturonic acid, usually at much lower rates than methyl esters (Manabe et al., 1973). The methanol is very volatile and can be distilled from 95-weight percentage alcohol product (Maiorella, 1985). The basic principle of toxicity of methanol is that it is metabolized primarily in liver and kidney by oxidation of formaldehyde and formic acid (Fig 2.8).

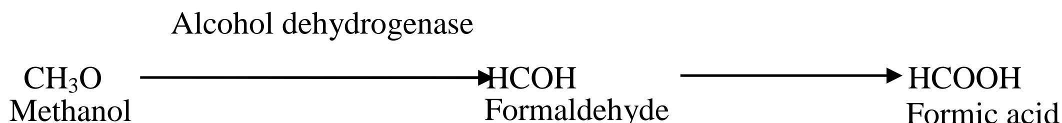


Fig 2.8 Conversion of methanol to formaldehyde and formic acid

2.20 Toxic effect

Major toxic effects are caused by formaldehyde and formic acid (Boing, 1987). The former is responsible for the damage of retinol cells that may cause blindness while the latter produces severe acidosis that may be eventually lead to death. A minor effect of methanol is depression of central nervous system (Boing, 1987). Occasionally, the severity of poisoning requires hemodialysis especially if the blood methanol concentration exceeds 500 mg/L or if the metabolic acidosis or neurological abnormalities proof refractory. Blood

methanol concentration below 50mg/L may be discounted. The fatal internal dose of methanol is 60-250ml. The exposure limit is 200mg/L.

2.21 Types of fermentation state

Fermentation has been widely used for the production of a wide variety of substances that are highly beneficial to individuals and industry. Over the years, fermentation techniques have gained immense importance due to their economic and environmental advantages. Ancient techniques have been further modified and refined to maximize productivity. This has also involved the development of new machinery and processes. Two broad fermentation techniques have been emerged as a result of this rapid development: Submerged fermentation (SmF) and Solid state fermentation (SSF). But the current research works have been going on in semi-solid state fermentation as well and it has been successfully commercialized for manufacturing biofuels (Machadoa *et al.*, 2013).

2.21.1 Solid substrate fermentation

Solid-state fermentation is a low cost fermentation process, particularly suitable to the agro-industrial residues as the substrates for the bioprocesses. It deals with the controlled growth of the microorganisms, mainly on the surface of water-insoluble substrates, in the presence of varying amounts of available water. Moo-Young *et al.* (1983) proposed the term Solid Substrate Fermentation(SSF) for all those processes where growth of microorganisms on moist solid particles in substrate beds, in which the interparticle spaces are filled with a continuous gas phase with a minimum of liquid water present in these interparticle spaces. The solid material serves as nutrient source as well as support for microbial growth. For bacteria, the growth tends to be confined to a biofilm on the particle surface. The moisture content is to be maintained sufficiently high in order to achieve water activity levels that are not limiting to microbial growth and should not exceed the water absorption capacity of the solid material (Castilho *et al.*, 2009).

Water activity is a thermodynamic parameter defined in relation to chemical potential of water. It represents the availability of water for reaction in the solid substrate. Water present in SSF system exists in a complex form within the solid matrix or as a thin layer adsorbed on the surface of the solid (Raimbault, 1998). Microbial growth will be critically affected by decrease of water activity (*a*w). Reduction of *a*w extends the lag phase, which

in turn decreases the specific growth rate and results in low amount of biomass (Oriol *et al.*, 1988). Bacteria generally require a higher *a*w when compared with fungi. The *a*w of the medium is a crucial factor for mass transfer of the water and solutes across the cell membrane, and the control of this parameter could be used to modify the metabolic production or excretion of a microorganism (Gervais, 1989).

The typical examples of SSF are the traditional fermentation, such as Japanese “koji”, Indonesian “tempeh” and French “blue cheese”. SSF processes have been proven to be particularly suitable for the production of the hydrolytic enzymes by the filamentous fungi, since they reproduce the natural living conditions of such fungi (Pandey *et al.*, 1999). The selection of an adequate solid substrate (support) for performing the solid-state cultivation is essential, since the success of the process largely depends on it.

The wide range of solid materials used in SSF can be classified into two major categories: inert materials, which only act as a solid support offering an attachment place for the microorganism, and natural materials, which not only function as solid support but also act as carbon source for the microorganism. These materials are typically starch or lignocellulose-based agricultural products or agro-industrial sources, such as grains and grain by-products (Pandey, 1992). The utilization of this kind of material helps in solving both the economic and the environmental problems caused by their disposal. SSF has found increased application using such materials (for example, straw, bran, oil cakes, etc.) for the production of antibiotics, surfactants, biocides and enzymes (Hernandez *et al.*, 1992; Pandey, 2001; Pandey *et al.*, 2000).

SSF process can also be classified based on whether the seed culture for fermentation is pure or mixed. In pure culture SSF, individual strains are used for substrate utilization and with mixed culture; different microorganisms are utilized for the bioconversion of agro-industrial residues simultaneously.

SSF offers several advantages in comparison to submerged fermentation (SmF), such as concentrated product formation, less water requirement, etc., but this system is susceptible to the water content, pH, oxygen gradients and accumulation of metabolic heat, making scaling up difficult.

Table 2.2 Comparison between Submerged state (SmF) and Solid state fermentation (SSF)

Factor	SmF	SSF
Substrates	Soluble substrates (sugars)	Insoluble substrates: starch, cellulose, pectin, lignin
Aseptic conditions	Heat sterilization and aseptic control	Vapour treatment, non- sterile conditions
Water	High volumes of water consumed and effluents discarded	Limited consumption of water; low *a*w. No effluent
Metabolic heating	Easy control of temperature	Low heat transfer capacity
Aeration	Limitation by soluble oxygen, high level of air required	Easy aeration and high surface exchange air/substrate
pH control	Easy pH control	Buffered solid substrates
Mechanical agitation	Good homogenization	Static conditions preferred
Scale up	Industrial equipment available	Need for engineering and new design equipment
Inoculation	Easy inoculation, continuous process	Spore inoculation, batch
Contamination	Risks of contamination for single-strain bacteria	Risk of contamination for low-rate growth fungi
Energy consideration	High energy consuming	Low energy consuming
volume of equipment	High volumes and high cost technology	Low volumes & low costs of equipment
Effluent & pollution	High volumes of polluting effluents	No effluents, less pollution

Source:(Sindhu *et al.*, 2015)

2.21.1.1 Role of water in solid state fermentation

- If the quantity of the water becomes insufficient and does not allow a good diffusion of solutes and gas, the cell metabolism slows, or can stop, because of a lack of substrates or through too high concentration of inhibitive metabolites in or near the cell.
- If the intracellular or extracellular quantity of water does not allow the maintenance of the functional properties of some enzymes, their inactivity creates a disequilibrium in the metabolic chain of the cells (Todd, 1972).
- In the same way, if the transfer of water induced by water stress leads to a denaturation of the mechanical structure of the plasmic membrane, all the properties of permeability and transport through the membrane are affected and the cell is then perturbed (Loecker *et al.*, 1978; Wolfe and Steponkus, 1983).

2.21.2 Semi-solid state fermentation

In semi-solid state fermentations the insoluble solid substrate is a solid porous matrix, which absorbs water with a relatively high water activity and also contains available carbohydrates, nitrogen sources and mineral nutrients. The attraction towards this type of culturing comes from its similarity to the natural way of life for many microorganisms (Couto *et al.*, 2001) and usage of starchy agricultural wastes makes the whole process more economical.

Part III

Materials and methods

3.1 Materials

Sorghum *(S. bicolor L. moench)* of white sweet variety was collected from local market of Makawanpur district. Fermentation starter was prepared using yeast *(Saccharomyces cerevisiae)* and mold *(Rhizopus oryzae)* isolated from traditional starter *(murcha)* in MYGP agar supplemented with copper. The traditional starter was confirmed to have shelf-life of not less than one year from the *murcha* seller to ensure that the sample was not too old (Karki, 1986).

Sorghum needed for fermentation were planted in early June in warm climates in the soil of Makawanpur district and harvested after ten months of plantation. Cane molasses needed for preliminary screening of yeast culture (Rai and Subba, 2003) was bought from Dharan market. Media needed for all microbiological works was obtained from Central Campus of Technology, Dharan laboratory and confirmed to be of Hi-Media®, India. All the necessary utensils, equipments and glasswares needed for the work was obtained from the campus.

3.2 Methods

3.2.1 Sample Preparation

After collection, the samples were packed in reclosable plastic pouches, taking care to avoid cross contamination and stored in bulk in the refrigerator until needed.

3.2.2 Testing of *murcha* sample

At first, trials were done to ascertain the quality of the cake purchased. This entailed rapid fermentation test in cooked rice (0.5 gram *murcha* powder per 100 gram cooked rice) for one week and tentative organoleptic appraisal of the product was done by panelist.

3.2.3 Mold isolation and identification

Having established the tentative quality of *murcha*, rapid screening of molds was carried out on MYGP agar using a modification of the method described (Malloch, 1997) and

(Harrigan and McCance, 1976). Small grains of *murcha* were embedded at 2-3 places in MYGP agar, keeping at least 1.5cm space between the kernels. Incubation was done at 25°C and observed daily for colony growth. The colonies were sub cultured on a fresh MYGP agar plate by spot culturing with the help of inoculating needle. Initial microscopic examination of the molds was done on the remaining portion of the colony by preparing tape culture (KC *et al.*, 2004). Molds were identified at the level of genera by combining microscopic examination (Malloch, 1997) and the KEYS (Malloch, 1997) given in Appendix E.

3.2.4 Testing of mold performance and propagation (*koji* preparation)

The test for the performance of molds was done by inoculating small lots of cooked rice and observing for the saccharification. Molds that exhibited saccharification property were propagated aseptically in sterile (autoclaved) wheat bran to produce *koji*. The process involved profuse sporulation of mold on MYGP agar (at 28-30°C, for 4-5 days); addition of a small amount (~ 10g) of autoclaved wheat bran on the plate; shaking of the plate (closed) to affect transfer of mold spores to the moist bran; aseptic transfer of bran *in toto* to 250g of autoclaved bran (50% moisture) previously kept in a sterile polythene bag; mixing; and aerobic growth in moist chamber at ~ 28-30°C and RH ~95% for 5-6 days.

RH was maintained by humidity chamber obtained from Central campus of Technology, Dharan laboratory. Because of the small size of the batch, spontaneous cooling was considered adequate for the removal of metabolic heat. The *koji* was taken out, mycelial network broken, allowed to dry in sun (to ~ 14% moisture content) spontaneously for another 5-6 days, packed, and stored at 5°C until needed. Asepsis was maintained throughout the operation (Rai, 2006).

3.2.5 Preservation of mold culture

The mold cultures were successfully preserved by sporulation in MYGP agar plates and storage at both refrigeration and room temperature. The cultures remained stable even after six months of storage found by (Rai, 2006). Mold cultures could also be maintained as dried *koji* in sterile wheat bran as shown in Fig. 3.1

Fig 3.1 Sterile wheat bran for propagation of isolated mold

3.2.6 Yeast isolation and identification

Fermentative yeasts were screened by inoculating fifteen gram of *murcha* in hundred millilitres of sterile cane molasses (TSS=10ºbrix and pH=4.5): incubation at 30ºC for a week (with occasional shaking for aeration) as defined by KC *et al.* (2004). Observation of gas formation and alcoholic smell denoted progress of the yeast fermentation. Microscopic examination by negative staining (for ascertaining the presence of yeast cells) was done and spread-plating on a series of five to six MYGP agar plates was done to sub-culture yeast colonies, followed by incubation at 30ºC for 1-3 days. Plate bearing well-isolated colonies was selected and the yeast isolates were characterized by the KEYS (Malloch, 1997) given in Appendix E.

3.2.7 Preservation of yeast isolates

The yeast isolates were successfully preserved by subculturing in MYGP plates. Rai (2006) has showed that the yeast culture of murcha sample could be preserved for six months under the same condition. They could also be maintained at room temperature (~ 30^0C) for 15 days in sterile molasses broth without any decrease in functional properties of the organism (Rai, 2006).

3.2.8 Testing of yeast performance

The performance of each yeast isolates was tested by pitching hundred millilitres of actively growing (3-day old) broth culture in 1 liter of sterile high-test cane molasses adjusted to16°brix. The pH of the medium was adjusted to 4.5 with 10% solution of citric acid before sterilization (autoclaving). Fermentation was carried out in cotton plugged 1-liter conical flask at 30°C until the TSS ceased to decrease further (Rai, 2006). The

characteristics of the isolates (e.g., flocculation, foaming) and the beer itself (smell, taste, etc) were noted to get an idea regarding their suitability in starter preparation.

3.2.9 Preparation of fermentation starters using mold and yeast isolates

Wheat bran *koji* was mixed with yeast isolates and propagated in moist rice flour as a carrier-cum-medium. Coarse variety of rice (500 gram) was washed and steeped for five hours in warm water (40ºC) that was acidified to pH 2.5 with citric acid solution. The low pH was used to discourage bacterial growth during steeping (Rai, 2006).

The steeped water drained and the rice was steamed cooked in autoclaved, followed by cooling to ambient temperature at 25^0C. Clean aluminum trays were used for cooling purpose rather than floor to avoid contamination. For every hundred gram of moist rice 0.5 gram of dry koji (~14% moisture content) and 0.5 ml of yeast sediment (obtained by propagating in lab as described for testing of yeast performance as described above) were added (Rai, 2006). The cultures were derived from the same *murcha* sample. The admixture was formed into stiff dough (~50% moisture, adjusted by adding distilled water). The dough was divided into small lumps and then gently patted into cakes (0.5 cm thick, 2-2.5 cm diameter). The cakes were placed (in single layer) on a large petri dish (20 cm diameter).

The dish was placed in the moist chamber maintained at RH ~ 95%. Each dish was finally covered with wet muslin cloth and was left for two days at 28^0C – 30^0C for fermentation. The swelling of cake, appearance of profuse mycelia and prevalence of sweet alcoholic smell were taken as indicator for the completion of fermentation. The dish was placed in sun for drying for 3 days until moisture content is reduced to ~5%. Then thus prepared starter culture was packed in air tight polythene bag and kept in room temperature. Fig 3.2 below entails the preparation of starter culture.

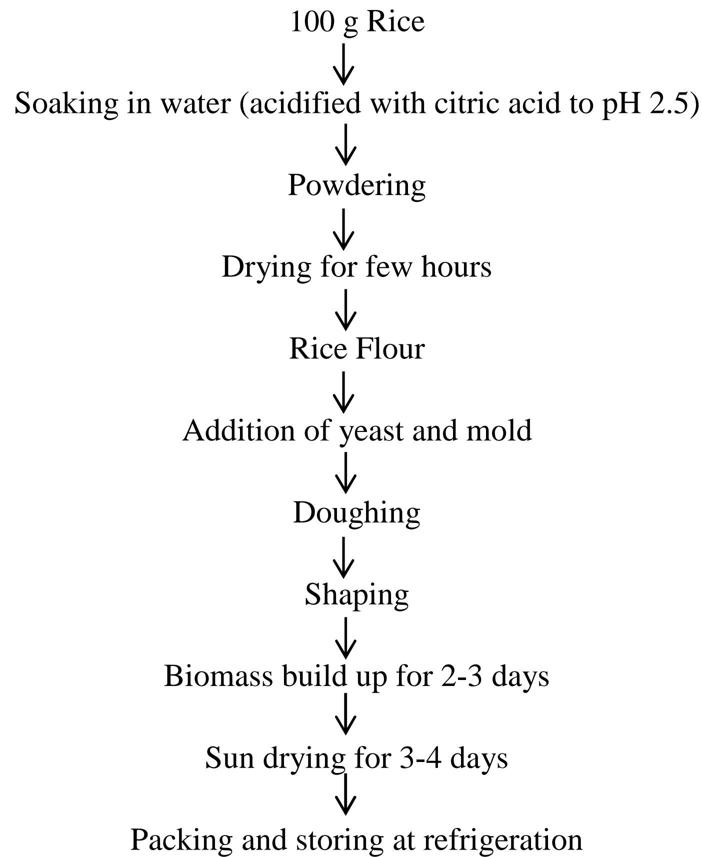


Fig. 3.2 Preparation of starter using rice flour

The starter types prepared in the laboratory are shown in Fig. 3.3. Incubation at 28ºC in a moist chamber (RH ~ 95%) for 48 hours produced highly characteristic puffy cakes overgrown by white mycelia. Longer incubation periods led to profuse spore formation, thereby making the cakes black.

Two-stage drying of *murcha* at temperatures below 55ºC (drying to around 25% moisture followed by moisture equilibration for overnight and a final drying to around 16%) in a cabinet dryer was found not to be detrimental as performed by (Rai, 2006). This is also the approximate temperature achieved during sun-drying. The shelf life of *murcha* has been reported to be ~ 1year (Karki, 1986) but this could not be ascertained in the present study because of time constraint.

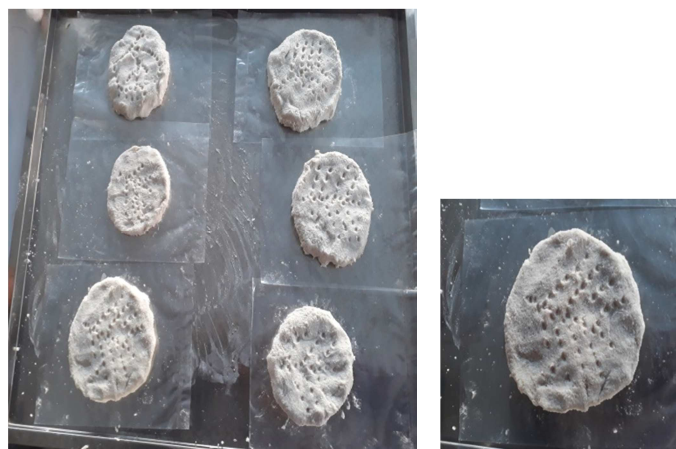


Fig. 3.3 Laboratory *murcha* prepared using mold and yeast isolates from local *murcha*

3.3 General method of preparation of *Jand*

3.3.1 Preparation of raw materials

Sorghum *(S. bicolor L. moench)* of white variety was cleaned, dehusked, winnowed and washed with water. The sorghum was steeped in water for two hours, washed again, cooked for forty-five minutes at 100 ºC, and cooled to room temperature.

3.3.2 Cooking of sorghum

The purpose of cooking sorghum grains is to convert the starch into amylose and to denature the protein in the sorghum. But, the cooking temperature was below the denaturation point of sorghum protein which is 105.8 ºC (Khattab *et al.*, 1972) in order to notice any change in protein solubility of sorghum protein after fermentation. *Aspergillus* spp. As well as *Rhizopus* spp. can then grow easily and its enzyme can react with the substrate rapidly. Steaming also destroys the contaminating micro-organisms. Cooking of sorghum grains is performed in an open conventional method in a large vessel, since gelatinization under pressure is not commercially economical. The cooked sorghum was spreaded onto a muslin cloth and allowed to air cooled.

3.3.3 Inoculation and fermentation

Murcha was added in the form of powder uniformly over the surface of the cooked and cooled mashes at the rate of 0.5% (w/w). After the addition of *murcha* powder it was mixed intimately with mash. After inoculation of *murcha* powder, the mashes were left for

biomass build up at room temperature for forty-eight hours at aerobic condition. After forty-eight hours, optimum biomass was seen in visible puffy colonies. The mashes (pH 4.5-5) were transferred to plastic jars which were previously cleaned for solid-state fermentation. For, semi-solid state fermentations, the biomass built-up sorghum mass was filled into plastic containers and previously boiled and cooled water was added to the containers at the rate of 50%, 75% and 100% (v/m) for semi-solid state $fermentations_1$, semi-solid state $fermentation_2$ and semi-solid state $fermentation_3$ respectively and kept at 28±1 ºC for twelve days for alcoholic fermentation and the days of completion of fermentation time was chosen on the basis of maximum acidity (% lactic acid) produced by bio-mass.

3.3.4 Preparation of sorghum *Jand*

For the preparation of sorghum *Jand*, 1.5 parts (by volume) of previously boiled and cooled water was added to each part (by weight) of biomass developed sorghum used for alcoholic fermentation. In the case of semi-solid fermented sorghum, the volume of water added previously during the start of alcoholic fermentations was subtracted from that of total volume of water to be added. The mixture was gently macerated, allowed to stand for twenty minutes and strained through muslin cloth with gentle pressing. The *Jand* (sorghum beer) obtained were subjected to chemical and organoleptic analyses. The overall experimental detail is given in fig. 3.4 below.

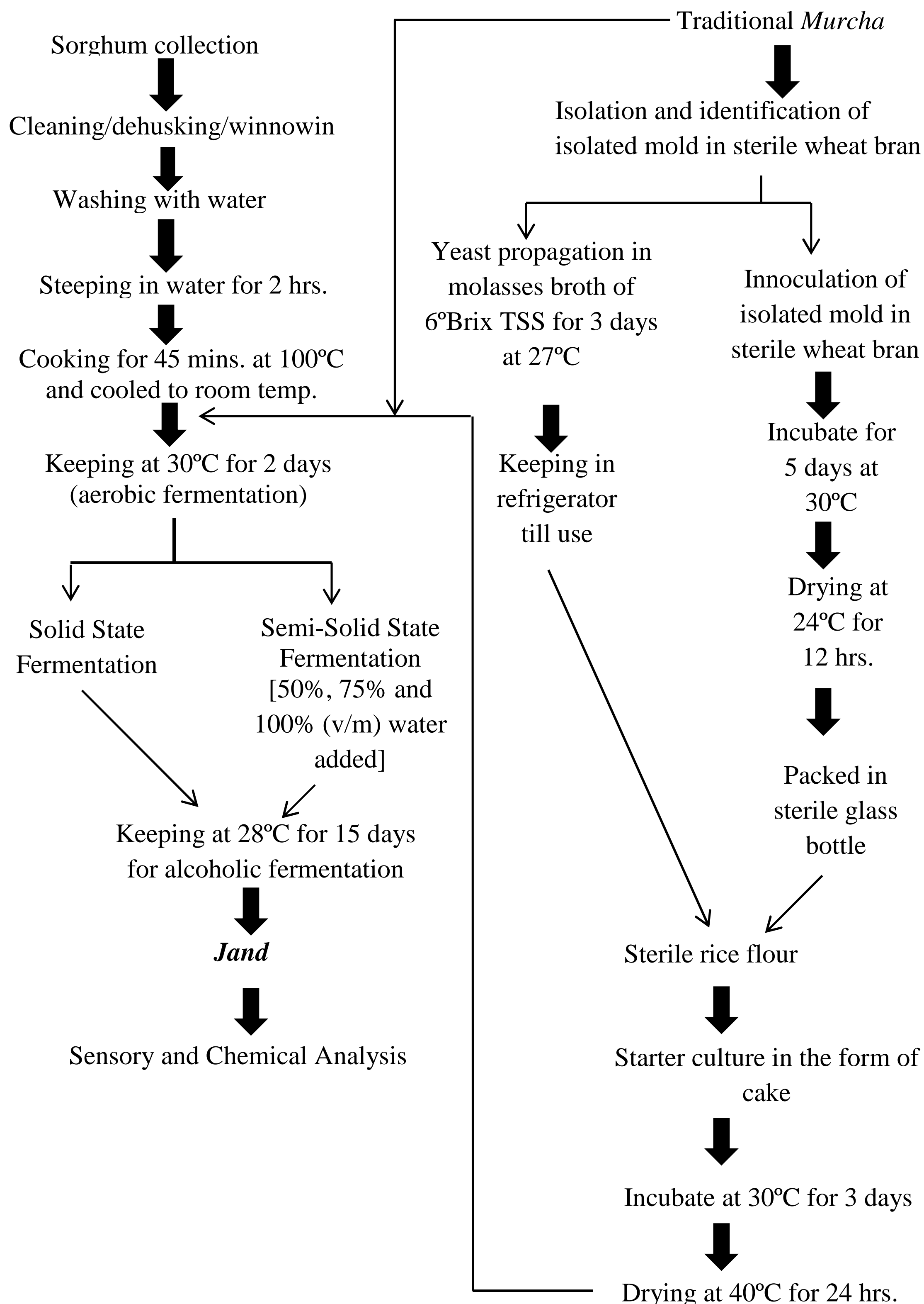


Fig. 3.4 overall experimental detail of preparation and analysis of sorghum *Jand*

3.4 Sensory evaluation and physicochemical analysis of the product

3.4.1 Sensory evaluation

Sensory evaluation of sorghum *Jand* was done using ten semi-trained panelists consisting of B.Tech (Food) students (age group 22-25) on a nine-point hedonic rating (9 = like extremely, 1 = dislike extremely) for taste, color, smell, and overall quality) of the *Jand*. They were asked to rate the product according to their liking or disliking following 9 points hedonic rating test as described (Ranganna, 2005).

3.4.2 Physico-chemical analysis

Fermented mash (*Jand*) was taken to determine its pH and moisture. Ten gram of the fermented mash was taken for the determination of acidity, in terms of lactic acid and it was index of the completion of fermentation time as well. Two hundred gram of fermented mash (after neutralization) was taken for distillation and the distillate was used for alcohol determination. After that, same distillate was divided equally (about 100 milliliters) for the determination of ester, aldehyde and methanol. The spent wash remained after distillation was taken for the determination of reducing sugar. For the analysis of change of protein solubility in sorghum after fermentation, the fermented mash of sorghum was taken for the determination of soluble protein.

3.4.2.1 pH

pH of the samples were determined by the digital pH meter of Labtronics^{TM} (Deluxe pH meter) of model LT-10 provided by Central Campus of Technology, Nepal and standardized with standard buffer at 25 ºC.

3.4.2.2 Alcohol Content

Alcohol content was determined by pycnometric method (AOAC, 1990) taking 200g of *jand* (mash) and the values were expressed in percentage (v/v).

3.4.2.3 Acidity

Acidity was determined on ten gram mash by titrimetric method (Ranganna, 2005) using 0.1*N* sodium hydroxide and the values were expressed in percentage (m/v) as lactic acid.

3.4.2.4 Methanol Content

Methanol contents of the samples were determined by colorimetric method described in (AOAC, 1990) and the values were expressed in terms of percentage (m/v).

3.4.2.5 Aldehyde Content

Aldehyde content was determined as per the method described in Nepal standard for country spirits (1984) and the values were expressed in gram per 100 liter of absolute alcohol as acetaldehyde.

3.4.2.6 Ester Content

Ester content was determined as per the method described in Nepal standard for country spirits (1984) and the values were expressed in gram per 100 liter of ethyl alcohol as ethyl acetate.

3.4.2.7 Dry matter content

Dry matter contents of the samples were determined as per the method described in (Pearson, 1971) and the values were expressed in percentage (m/v).

3.4.2.8 Reducing sugar and starch content

Reducing sugars and non-reducing carbohydrates were determined by Lane and Eynon method as described in (Ranganna, 2005) on spent wash recovered after distillation of alcohol. The conventional Carrez solution was used along with 0.3*N* barium hydroxide solution for clarifying the sample. Ten milliliters each Carrez solution and barium hydroxide was used for every fifty milliliters of the extract. The values of reducing sugar were expressed as percentage (m/v) as dextrose and starch contents was determined in terms of percentage (dry basis).

3.4.2.9 Protein solubility

Protein solubility of raw sorghum grains were determinated in triplicate by determining soluble proteins with conventional Kjeldahl method as per (AOAC, 1990). Soluble proteins were calculated in tems of percentage (dry basis) of total sorghum protein. Protein solubilities of *Jand* from different variations of fermentation starters and fermentation

medium (state) were determined in the similar way and percentage calculated in terms of total initial sorghum protein in raw sorghum grains.

3.4.3 Data analysis

The work was carried out in duplicate (starting from the research culture) while the analyses were carried out in triplicate. Data on microbial - and physicochemical parameters of traditional starter and isolated pure culture starter were tabulated for comparison and a descriptive treatment given. Data on sensory quality of Sorghum *Jand* from different starters (laboratory and traditional) and fermentation states (solid state and 3 different semi-solid states) were statistically processed by One Way Analysis of Variance (ANOVA). The physicochemical properties of *Jand* were similarly processed for ANOVA. Means of the data were compared using LSD (least significant difference) method at 5% level of significance.

Part IV

Results and discussion

Sorghum(*S. bicolor L. moench*) of white variety were taken from Makawanpur district which was planted in early June in warm climates and harvested after 10 months of plantation. Mold of *Rhizopus spp.* and *Saccharomyces cereviseae* yeast were isolated from traditional fermentation starter (*murcha*) in MYGP agar and propagated in suitable medium and environment of rice bran and molasses respectively for mold and the yeast. Pure-cultured starter was developed in the form of cake using rice flour and isolated culture. Saccharification and fermentative performance of the developed pure-culture cake and traditional *murcha* were done in cooked rice and sorghum in a beaker simultaneously and with positive results given by regular panelists, both types of starters were taken in fermentation of sorghum. Fermentation conditions for sorghum were given similar for both types of starters and the fermentation conditions were of solid and semi-solid state, where semi-solid state were of 50, 75 and 100% water addition. Sensory analysis was done with all 8 samples of *Jand* from solid and three semi-solid fermentation states and two types of starters by semi-trained panelists, while chemical analysis were done to compare between two fermentation starters and between different fermentation states. To find out change in protein solubility of sorghum (very low in raw sorghum) after fermentation by varying, fermentation starters and states was given priority above all.

4.1 Screening of saccharifying molds and fermenting yeasts

Molds were successfully screened from *murcha* sample by using a modification of the screening techniques described by Malloch (1997) and KC *et al.* (2004). It was found that screening showed the dominance of a single–type, morphologically distinct mold colony. There was no difference with respect to colour and abundance of spores in the culture when isolated in MYGP agar medium. The inoculation of the rice substrate with *murcha* samples gave the single type mold colonies that ultimately developed into profuse spores.

4.1.1 Mold identification

The mold isolates were characterized by microscopic examination and colony characteristics, to be species of *Rhizopus* according to description of molds given by

(Aneja, 1996). The KEYS to the mold genera (Malloch, 1997) referred for the identification are given in Appendix E. The microscopic isolated molds are shown in Fig. 4.1.

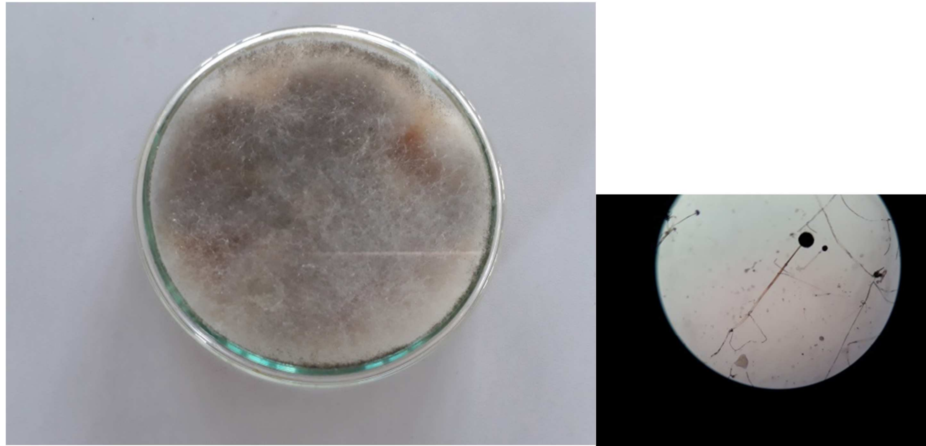


Fig. 4.1 48 hours older photomicrograph of molds isolated from starter culture (traditional *murcha*) (100X)

4.1.2 Screening and identification of yeast

Fermentative yeasts were screened by the rapid screening technique described by Subba *et al.* (2005)and KC *et al.* (2004) which yield single type fermentative yeasts with similar morphological and fermentation characteristics. The yeast isolates from *murcha* samples were identified to be the strains of *Saccharomyces cerevisiae*. The KEYS used for the identification are given in appendix E.

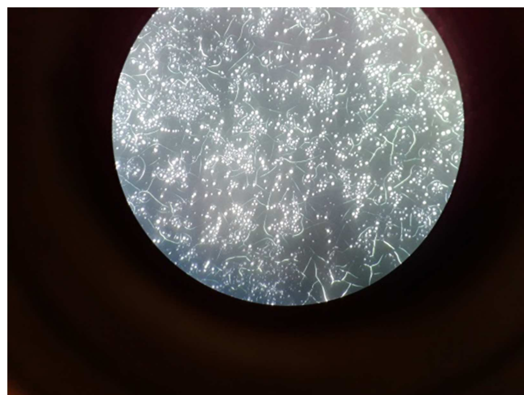


Fig. 4.2 36 hours older yeast colony of *S. cerevisiae* identified

4.2 Proximate composition of sorghum (*S. bicolor L. moench*) of white variety

Table 4.1 Proximate composition of sorghum

Parameters	Average value
Moisture content (%)	10±1.24%
Crude fat (%db)	2.367±0.2%
Crude Fiber (%db)	1.93±0.4%
Total Ash (%db)	1.67±0.4%
Crude protein (%db)	13.39±0.6%
Starch (%)	58.57±4%
Protein solubility (%m/m)	28.4±1.5%

*The values in the table are the mean of the triplicates ± standard deviation

The proximate compositions of sorghum grain were found to be similar as given by Alais and Linden (1991) but the crude fiber content was found to be lower than that of proximate data reported by Alais and Linden (1991). All the proximate data were closer to finger millet except soluble protein fractions and total carbohydrate content. Adebiyi *et al.* (2005) reported moisture content, ash content, fat, crude fiber, protein and carbohydrate of unhydrolyzed sorghum to be 10.66%, 1.98%, 3.35%, 2.25%, 9.35% and 72.41% respectively. Although, protein content and starch of sorghum reported by Adebiyi *et al.* (2005) did not match with this work, other results were closer to that of obtained results. Similarly, R. W. Jones (1970)reported protein, starch, fat, crude fiber and ash content of sorghum hybrid OK612 were 11.6%, 75.9%, 3.3%, 1.9% and 1.3% respectively in dry basis which were closer to the results obtained in this work.

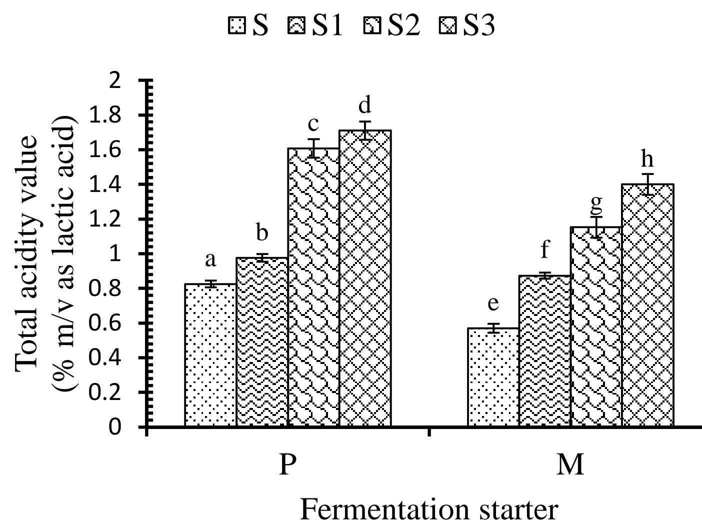
4.3 Chemical analysis of prepared *Jand*

Sorghum was fermented by using traditional *murcha* and pure-cultured starters under solid state fermentation and semi-solid state fermentation with 50%, 75% and 100% water added. The sorghum *Jand* was analyzed for acidity, pH, alcohol content, ester content, aldehyde content, methanol, dry matter content, reducing sugar and protein solubility.

Similar conditions of fermentation were provided to cooked sorghum without addition of water and pH adjusted for solid state fermentation, by using two starters: traditional *murcha* and pure-cultures starter (laboratory *murcha*) at the rate of 0.5% (w/w). After the completion of the fermentation (12 days with 2 days of extra aerobic period for biomass development at 28-30ºC), chemical analysis were performed. Fig. 4.6 shows the average scores. The effects of fermentation starters and fermentation states on chemical and sensory properties of sorghum *Jand* are given below.

4.3.1 Effect of fermentation starters and fermentation states on chemical properties of sorghum *Jand*

4.3.1.1 Effect on total acidity



*Bars having similar letter are not significantly different by LSD at p=0.05

Fig. 4.3 Effect of fermentation states on the total acidity of sorghum *Jand*

The samples were coded P and M denoting pure cultured starter and traditional *murcha* under fermentation states S, S1, S2 and S3 for solid state, semi-solid state (50%), semi-solid state (75%) and semi-solid fermentation state (100%) respectively.

The average total acidity of *Jand* obtained from traditional *murcha* was found to be 0.57, 0.87, 1.15 and 1.4% for solid state and semi-solid state with 50, 75 and 100% water addition respectively. While, the acidity value of *Jand* from pure-cultured starter was

found to be 0.83, 0.98, 1.61 and 1.71% for solid state and semi-solid state with 50, 75 and 100% water addition respectively. Statistical analysis showed that fermentation conditions had a significant effect ($p<0.05$) on the total acidity of sorghum *Jand* prepared by using traditional and pure-culture of yeast and mold. Increasing water addition significantly increased total acidity in both types of fermentation starters. LSD indicated that fermentation starter had a significant effect ($p<0.05$) on the total acidity content under the same fermentation condition.

According to Shrestha (1985), rice fermented for 8 days, using *murcha* had total acidity of 2.54% (m/v). According to Subba (1985), fermented mash of *kodo* for 7 days, using *murcha* had total acidity of 2.13% (m/v). These values were far higher than the values obtained in this work. Mongar and Rai (2005) reported that the total acidity (% m/v as lactic acid) contents in millet *Jand* fermented by using *murcha* was 0.6%. The data obtained in this study were found to be lower for traditional *murcha* and higher for pure culture than those reported by Mongar and Rai (2005). The value may be lower for traditional *murcha* due to inconsistency in the quality of *murcha* as it contains various molds, yeast and bacteria. The high acid content for *Jand* from pure culture may be due to the production of lactic acid, fumaric acid by organism i.e., yeast, mold and even bacteria such as *pediococcus pentoceus, Lactobacillus plantaram*. Due to lack of other foreign yeasts and molds other than *Rhizopus spp*. and *Saccharomyces cerevisiae*, bacteria will find more place to grow and produce lactic and acetic acid from their life cycle as well which may contribute to increase acidity value in case of pure fermentation starter.

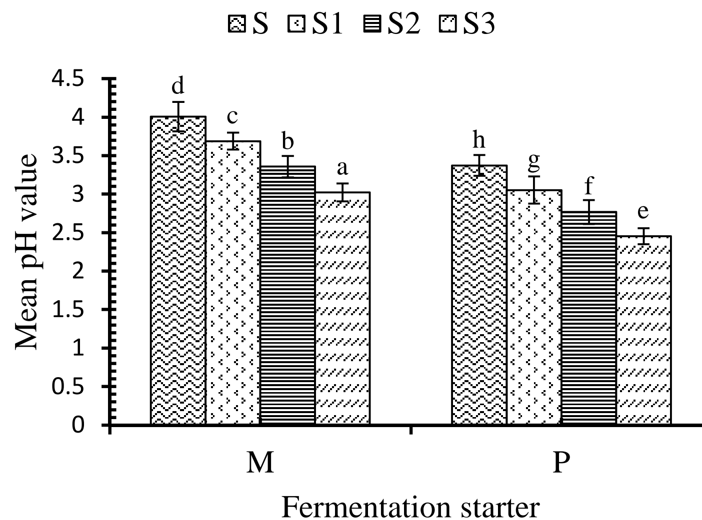
Semi-solid state fermentation showed significant effects ($p<0.05$) on the total acidity of the sorghum *Jand* (sorghum brews) compared to solid state. The significant effect was shown by both traditional and pure-cultured starters. Similar results for the increase in the total acidity under semi-solid state fermentation were also reported by Karki and Kharel (2010). Similar results of total acidity in traditionally prepared *Jand* from millet were also reported by Thapa and Tamang (2004).

In lactic acid production, which is oldest microbial metabolites, conversion of starch to lactic acid takes place (Yun *et al.*, 2003). In semi-solid state fermentations, the insoluble solid substrate is a solid porous matrix, which absorbs water with a relatively high water activity and also contains available carbohydrates, nitrogen sources and mineral nutrients

(Naveena *et al.*, 2004). Due to sufficient water activity, bacteria such as *pediococcus pentoceus, lactobacillus plantaram* can grow easily in semi-solid state fermentation which may have added to high acidity count by the production of lactic and acetic acid as their metabolites (side product of Embden-Meyerhof fermentation cycle).

Other reasons for increasing acidity may be due to the oxidation of aldehyde to form acetic acid. So, it can be said that aldehyde present in the samples were converted into acetic acid. Oxidation of ethyl alcohol to acetic acid may be reason for increase in acidity as well. During fermentation, the vessels were not completely filled with potato mash, so it was not possible to maintain the anaerobic condition. Acidity plays an important role in the sensory, which is seen in sensory results clearly. The high acidity of the product impairs the other quality of the product.

4.3.1.2 Effect on pH



*Bars having similar letter are not significantly different by LSD at p=0.05

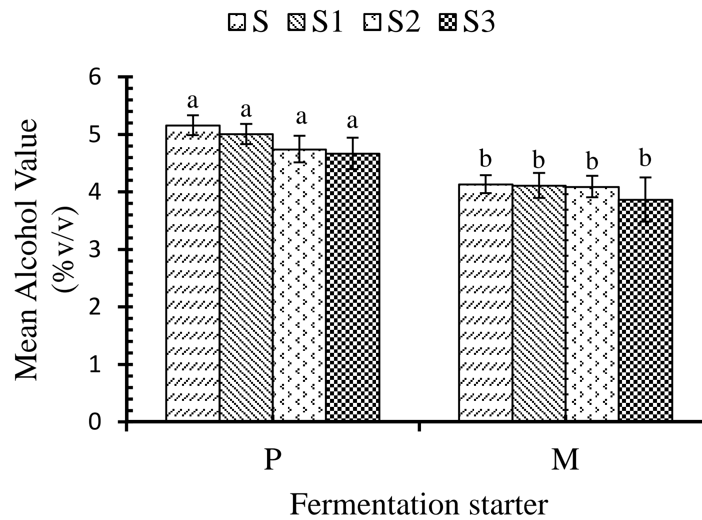
Fig. 4.4 Effect of fermentation states on the pH value of sorghum *Jand*

The samples were coded P and M denoting pure cultured starter and traditional *murcha* under fermentation states S, S1, S2 and S3 for solid state, semi-solid state (50%), semi-solid state (75%) and semi-solid fermentation state (100%) respectively.

The average pH values of *Jand* obtained from traditional *murcha* were found to be 4.01, 3.69, 3.36 and 3.02 for solid state and semi-solid state with 50, 75 and 100% water addition respectively. While, the acidity value of *Jand* from pure-cultured starter was found to be 3.37, 3.05, 2.77 and 2.45 for solid state and semi-solid state with 50, 75 and 100% water addition respectively. Statistical analysis showed that fermentation conditions had a significant effect ($p<0.05$) on the pH value of sorghum *Jand* prepared by using traditional and pure-culture of yeast and mold. Increasing water addition significantly decreased pH value in both types of fermentation starters. LSD indicated that fermentation starter had a significant effect ($p<0.05$) on the pH value under the same fermentation condition.

According to NS standard beer should have a pH from 3.00 to 4.80. The pH value seems to be within the range, more for traditional *murcha* than the pure-cultured starter. The decrease in pH for the pure culture might be due to the formation of higher amount of ester than that of market *murcha.* Result on pH can be compared with Subba (1985), but he reported higher values of pH in traditionally prepared millet *Jand*. Results of pH from this study resembles with the work of Karki and Kharel (2010)in finger millet *Jand* prepared using pure culture in both solid and semi-solid states; but found higher than the result shown by Chaudhary (2013) in solid state fermentation.

4.3.1.3 Effect on alcohol content



*Bars having similar letter are not significantly different by LSD at p=0.05

Fig. 4.5 Effect of fermentation states on the alcohol content value of sorghum *Jand*

The samples were coded P and M denoting pure cultured starter and traditional *murcha* under fermentation states S, S1, S2 and S3 for solid state, semi-solid state (50%), semi-solid state (75%) and semi-solid fermentation state (100%) respectively.

The average alcohol content of *Jand* obtained from traditional *murcha* were found to be 4.13, 4.11, 4.09 and 3.873% for solid state and semi-solid state with 50, 75 and 100% water addition respectively. While, the alcohol content of *Jand* from pure-cultured starter was found to be 5.16, 5.01, 4.74 and 4.67% for solid state and semi-solid state with 50, 75 and 100% water addition respectively. Statistical analysis showed that fermentation starters had a significant effect ($p<0.05$) in alcohol content of the *Jand*. But, increasing water addition had no significant difference ($p<0.05$) in alcohol content in both types of fermentation starters. LSD indicated that fermentation starter had a significant effect ($p<0.05$) on the alcohol content under the same fermentation condition.

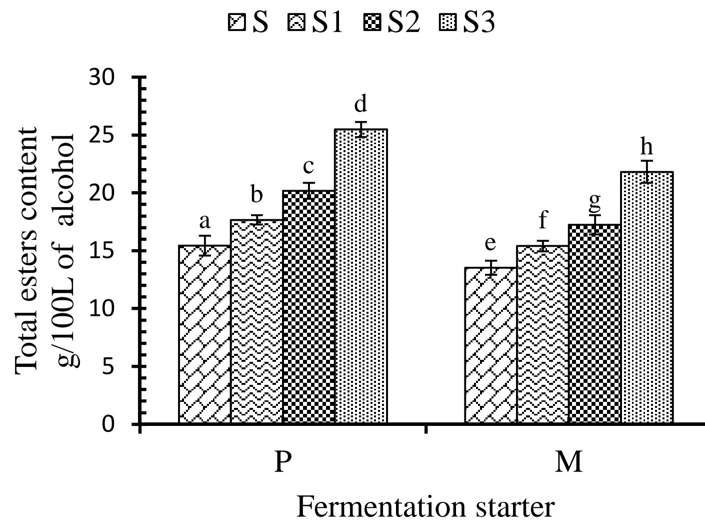
According to Karki (1986), *Jand* contain about 4-10% (v/v) alcohol. The alcohol content of *Jand* obtained in the present work was found to fall within this range. The lower alcohol content in traditional *murcha* may be due to the insufficient saccharification of sorghum starch, which may be due to the lack of saccharifying enzyme produced by mold in

murcha. Higher alcohol content in pure culture may be due to higher saccharification power of mold.

The values of alcohol content were lower than the values shown by Karki and Kharel (2010) in both solid and semi-solid state fermentation of finger millet. Also the values are lower than the values shown by Subba (1985) in solid state fermentation. But, alcohol content is comparable and similar to the study of Mongar and Rai (2005) in millet *Jand* fermented using *murcha* in solid state fermentation.

Cai and Nip (1990) reported analogous effect of semi-solid state fermentation on glucose in taro fermentation using starter containing *R. tankinensis, R. oryzae, R. chinensis and S. cerevisiae*, but contrary to our finding, they reported a significantly higher yield of alcohol in semi-solid fermentation than that of solid state fermentation. Low alcohol content in the product may be due to much amount of water used in comparison to the level of substrates as per recipe is concerned. Also, it may be due to the chemical constituents of *murcha,*which might have inhibitory effect to micro-organisms (Dangol, 2006).

4.3.1.4 Effect on total ester content



*Bars having similar letter are not significantly different by LSD at p=0.05

Fig.4.6 Effect of fermentation states on the esters content of sorghum *Jand*

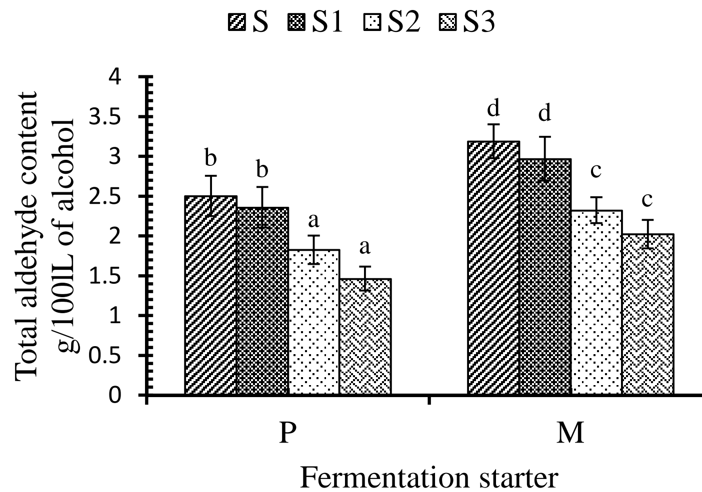
The samples were coded P and M denoting pure cultured starter and traditional *murcha* under fermentation states S, S1, S2 and S3 for solid state, semi-solid state (50%), semi-solid state (75%) and semi-solid fermentation state (100%) respectively.

The average ester content of *Jand* obtained from traditional *murcha* were found to be 13.53, 15.4, 17.24 and 21.81 for solid state and semi-solid state with 50, 75 and 100% water addition respectively. While, the ester content of *Jand* from pure-cultured starter was found to be 15.44, 17.66, 20.17 and 25.48 for solid state and semi-solid state with 50, 75 and 100% water addition respectively. Statistical analysis showed that fermentation conditions had a significant effect ($p<0.05$) on the esters content of sorghum *Jand* prepared by using traditional and pure-culture of yeast and mold. Increasing water addition significantly increased esters content in both types of fermentation starters. LSD indicated that fermentation starter had a significant effect ($p<0.05$) on the esters content under the same fermentation condition.

Effect of solid state fermentation can be compared with other research works. According to Engan (1981), ethyl acetate was found in between 8.2-47.6 g/100lit. in beer. The average ester content (as ethyl acetate) were found to be 15.84, 7.04, 22.88, 22.88 g/100lit. for wheat, millet, rice and maize *Jand.* The ester content of the *Jand* as ethyl acetate is 2-fold greater than that of the millet and comparable to that of wheat in solid state. Ester content effects the sensory property of the *Jand,* which were clearly shown by the sensory scores to be discussed later.

The average ester contents (as ethyl acetate) were found to be 7.04, 6.89, 7.55 and 6.93 g/100lit of absolute alcohol for *murcha* treated malted and unmalted and for *koji* treated malted and unmalted millet respectively (Upadhyaya, 2005). The values seem to be lower than that of this study. Higher values of esters will impair sensory quality (Berry and Chamberian, 1986); which is seen in the sensory results of *Jand,* especially in semi-solid state fermented products, which were quite unacceptable in terms of smell and flavor with increasing water content. Lower values of aldehydes for constant ethyl alcohol may have added higher ester values in semi-solid state fermentation with increasing water addition.

4.3.1.5 Effect on total aldehyde content



*Bars having similar letter are not significantly different by LSD at p=0.05

Fig.4.7 Effect of fermentation states on the aldehyde content of sorghum *Jand*

The samples were coded P and M denoting pure cultured starter and traditional *murcha* under fermentation states S, S1, S2 and S3 for solid state, semi-solid state (50%), semi-solid state (75%) and semi-solid fermentation state (100%) respectively.

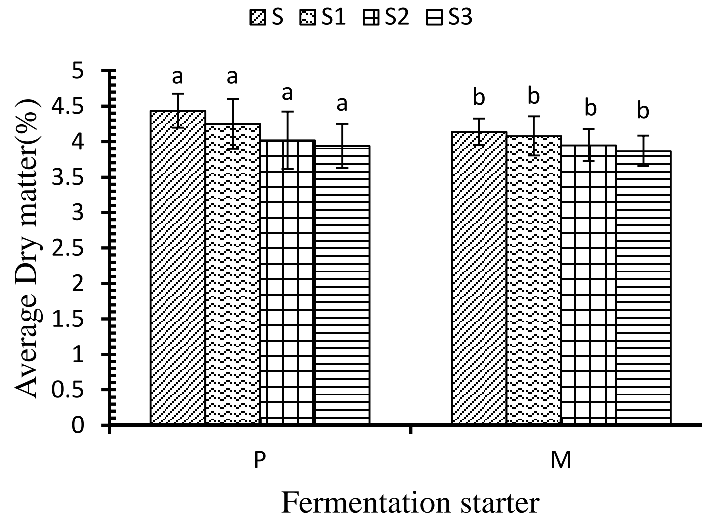
The average aldehyde content of *Jand* obtained from traditional *murcha* were found to be 3.19, 2.97, 2.32 and 2.02 for solid state and semi-solid state with 50, 75 and 100% water addition respectively. While, the aldehyde content of *Jand* from pure-cultured starter was found to be 2.50, 2.36, 1.83 and 1.46 for solid state and semi-solid state with 50, 75 and 100% water addition respectively. Statistical analysis showed that fermentation conditions had a significant effect ($p<0.05$) on the aldehyde content of sorghum *Jand* prepared by using traditional and pure-culture of yeast and mold. Increasing water addition significantly decreased aldehyde content in both types of fermentation starters. LSD indicated that fermentation starter had a significant effect ($p<0.05$) on the pH value under the same fermentation condition. But, there were no significant difference in the aldehyde contents in solid and semi-solid state with 50% water addition using both types of fermentation starters.

Aldehyde lowers the flavor of the alcoholic product. Value of aldehyde (as acetaldehyde) is lower for pure-cultured starter which may contribute to flavor of the product (and, is justified by the sensory scores), or, there may be conversion of aldehyde to acetic acid. The low value of aldehyde of semi-solid state fermentation and significantly increased value of acidity by them justifies latter statement. The lower aldehyde content in the pure culture may be also due to the reason of yeast, as its formation is also dependent on the yeast strain. So, it is said that there should be good quality of yeast in *murcha.* The mean value of aldehyde content of alcohol made from sweet potato using traditional *murcha* at the concentration of 0.5%, 1.0%, 1.5% and 2.0% were found to be 6.6, 5.5, 2.2 and 4.4 g/100 lit. according to Schweinberger *et al.* (2013) respectively and the result obtained in this work correlate with the above work. According to Engan (1981), acetaldehyde was found in between 0-24.4 (mg/lit.).

The average aldehyde contents (as acetaldehyde) were found to be 5.61, 0.61, 0.33 and 1.50 g/100 lit. for wheat, millet, rice and maize *Jand* respectively (Upadhyaya, 2005). The values of aldehyde in sorghum *Jand* are not comparable to that of millet *Jand,* but are comparable to the maize in solid state fermentation. Both esters and aldehyde values are seen uncomparable between sorghum and millet *Jand* (commonly consumed in Nepal).

Acetaldehyde reacts with ethyl alcohol to form acetal, a substance with a strong aldehyde like flavor (Amerine *et al.*, 1967). Acetaldehyde is thus quantitatively the most significant compound of this group as ethanol is the dominant alcohol formed during alcoholic fermentation. Since, aldehydes have flavor threshold two to three orders of magnitude below the alcohols; the quantity of aldehydes (mg/100 liter) in this work is considerably low to effect sensorial property of the sorghum *Jand.* The average aldehyde content (as acetaldehyde) were 7.04, 6.89, 7.55 and 6.93 for murcha treated malted and unmalted and for koji treated malted and unmalted millet respectively (Upadhyaya, 2005). These values are contrary to that of this study of fermentation of sorghum.

4.3.1.6 Effect on dry matter content



*Bars having similar letter are not significantly different by LSD at p=0.05

Fig.4.8 Effect of fermentation states on the dry matter content of sorghum *Jand*

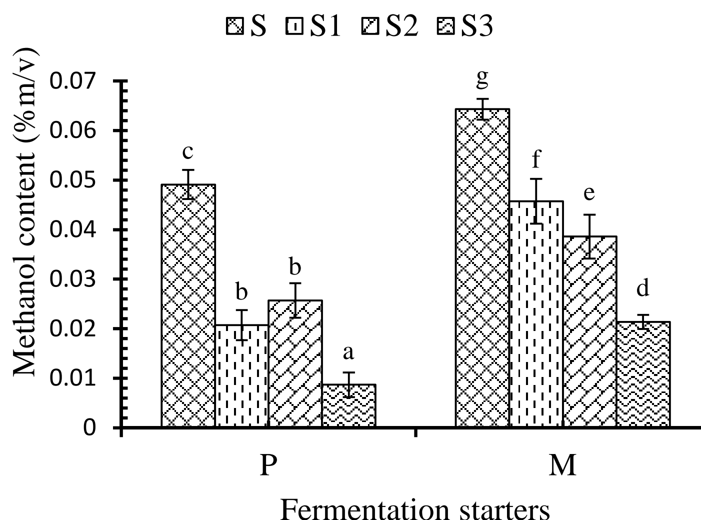
The samples were coded P and M denoting pure cultured starter and traditional *murcha* under fermentation states S, S1, S2 and S3 for solid state, semi-solid state (50%), semi-solid state (75%) and semi-solid fermentation state (100%) respectively.

The average dry matter content of *Jand* obtained from traditional *murcha* were found to be 4.12, 4.08, 3.95 and 3.87% for solid state and semi-solid state with 50, 75 and 100% water addition respectively. While, the dry matter content of *Jand* from pure-cultured starter was found to be 4.44, 4.25, 4.02 and 3.94% for solid state and semi-solid state with 50, 75 and 100% water addition respectively. Statistical analysis showed that fermentation starters had a significant effect ($p<0.05$) in alcohol content of the *Jand*. But, increasing water addition had no significant difference ($p<0.05$) in alcohol content in both types of fermentation starters. LSD indicated that fermentation starter had a significant effect ($p<0.05$) on the alcohol content under the same fermentation condition. However, the values are lower than the dry matter content (%) of yam *Jand* as found by Chaudhary (2013) which was 6.54% for traditional *murcha* and 5.12% for pure cultured starter.

Moisture contents and dry matter contents of sorghum *Jand* were not affected by semi-solid state fermentation ($p>0.05$) in comparison with the solid state. Presence of volatile

compounds in increasing water content of semi-solid state fermentation may have resulted in similar dry matter contents as that of solid state fermentation. The values of dry matter content is analogous to study of solid and semi-solid state fermentation on finger millet by Karki and Kharel (2010); but values of dry matter content in this study is lower than that of study of yam *Jand* prepared using traditional and pure culture by Chaudhary (2013).

4.3.1.7 Effect on methanol content



*Bars having similar letter are not significantly different by LSD at p=0.05

Fig.4.9 Effect of fermentation states on the methanol content of sorghum *Jand*

The samples were coded P and M denoting pure cultured starter and traditional *murcha* under fermentation states S, S1, S2 and S3 for solid state, semi-solid state (50%), semi-solid state (75%) and semi-solid fermentation state (100%) respectively.

The average methanol content of *Jand* obtained from traditional *murcha* was found to be 0.06433, 0.04567, 0.0386 and 0.02137% for solid state and semi-solid state with 50, 75 and 100% water addition respectively. While, the methanol content of *Jand* from pure-cultured starter was found to be 0.04913, 0.03067, 0.02567 and 0.00867% for solid state and semi-solid state with 50, 75 and 100% water addition respectively. Statistical analysis showed that fermentation conditions had a significant effect ($p<0.05$) on the total acidity of sorghum *Jand* prepared by using traditional and pure-culture of yeast and mold. Increasing water addition significantly decreased methanol content in both types of fermentation

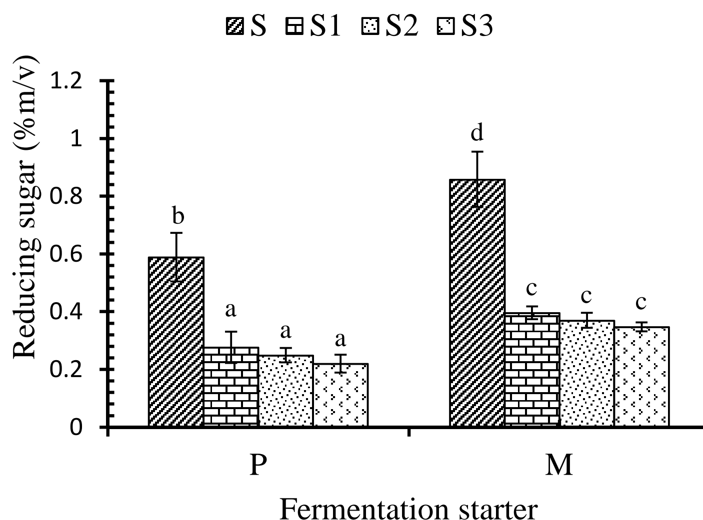
starters. LSD indicated that fermentation starter had a significant effect ($p<0.05$) on the total acidity content under the same fermentation condition. But, there was no significant difference in the methanol content in semi-solid states with 50 and 75% water addition using pure-culture starter.

According to Austin (1968), the methanol content of beer samples was found within the range of 0.01 to 0.35 (%m/v). The methanol content of both the samples was found within the range of beer.

The methanol arises by demethylation of pectin by pectin esterase enzyme (Boing, 1987). Yeast do not form an enzyme capable of hydrolyzing pectin and consequently the reaction does not commonly occur in cereal fermentation. But, pectin esterase is abundant in fungi. The variety of fungi colonies in traditional *murcha*, thus gave high methanol reading than that of pure- cultured fermentation starter.

In solid state fermentation, the results were contrary to that of yam *Jand* studied by Chaudhary (2013) in terms of methanol content, as the values in this study were higher than that of methanol content in yam *Jand*. Due to lack of relevant information, methanol content in fermented cereals under semi-solid state could not be compared, but the values obtained in this study lie within the range of beer according to Austin (1968). The lower values of methanol content in increasing water content of semi-solid state may be due to the lack of sufficient pectin esterase enzyme produced by mold and bacteria, when compared to that of solid state fermentation

4.3.1.8 Effect on reducing sugar



*Bars having similar letter are not significantly different by LSD at p=0.05

Fig.4.10 Effect of fermentation states on the reducing sugar of sorghum *Jand*

The samples were coded P and M denoting pure cultured starter and traditional *murcha* under fermentation states S, S1, S2 and S3 for solid state, semi-solid state (50%), semi-solid state (75%) and semi-solid fermentation state (100%) respectively.

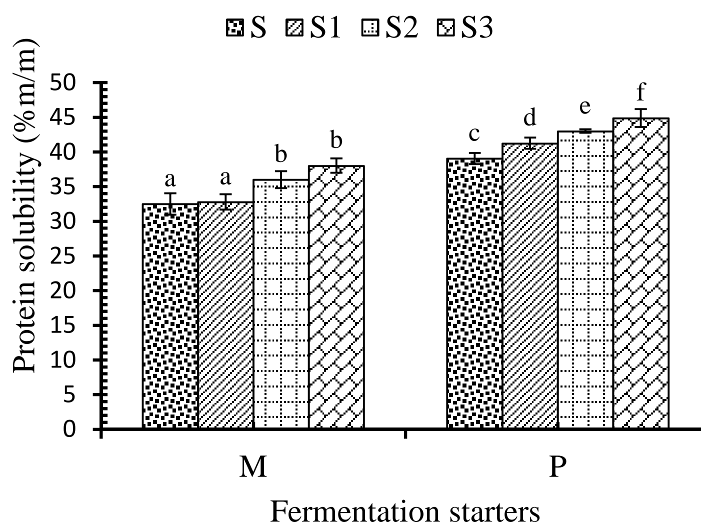
The average reducing sugar of *Jand* obtained from traditional *murcha* were found to be 0.86, 0.40, 0.37 and 0.35% for solid state and semi-solid state with 50, 75 and 100% water addition respectively. While, the reducing sugar of *Jand* from pure-cultured starter was found to be 0.59, 0.28, 0.25 and 0.22% for solid state and semi-solid state with 50, 75 and 100% water addition respectively. Statistical analysis showed that fermentation starters had a significant effect ($p<0.05$) in reducing sugar of the *Jand*. But, increasing water addition had no significant difference ($p<0.05$) in reducing sugar in both types of fermentation starters. LSD indicated that fermentation starter had a significant effect ($p<0.05$) on the reducing sugar under the same fermentation condition. However, there were significant differences in reducing sugar between solid and three semi-solid states using both types of fermentation starters.

Rai (2006) investigated from his study to give the values of reducing sugar of 0.92% and 1.02% for millet *jand* prepared using pure-cultured starter and traditional *murcha* in solid

state fermentation. The value obtained from traditional starter in solid state was near to this, but value from pure-culture starter was far lower. The low value of reducing sugar may be due to low saccharifying power of mold to the available carbohydrate or may be due to high kinetics of alcohol fermentation from available simple sugars (Subba, 1985). From the anatomical study of grain structure of sorghum (described in part II), it is understood that starch digestibility gets improved after cooking, but not enough as that of other cereals like millet, maize etc. which can explain low value of reducing sugar. Similarly, low value of reducing sugar by pure-cultured starter than the traditional one can be explained due to low degree of production of amylase enzyme by *Rhizopus spp.*

This result supports the findings of Karki and Kharel (2010) in his study, regarding reducing sugar in solid and semi-solid state fermentation of finger millet; but still there is lack of relevant information regarding sudden fall in reducing sugar content when fermented under semi-solid state. According to Mongar and Rai (2005), the reducing sugar (%m/v as dextrose) in millet *Jand* fermented by using *murcha* was 1.52% which was higher than the values obtained in this study under both solid and semi-solid state fermentation.

4.3.1.9 Effect on protein solubility



*Bars having similar letter are not significantly different by LSD at p=0.05

Fig.4.11 Effect of fermentation states on the protein solubility of sorghum *Jand*

The samples were coded P and M denoting pure cultured starter and traditional *murcha* under fermentation states S, S1, S2 and S3 for solid state, semi-solid state (50%), semi-solid state (75%) and semi-solid fermentation state (100%) respectively.

The average protein solubility of *Jand* obtained from traditional *murcha* was found to be 32.5, 32.78, 36.01 and 38.08% for solid state and semi-solid state with 50, 75 and 100% water addition respectively. While, the protein solubility of *Jand* from pure-cultured starter was found to be 39.05, 41.26, 43 and 44.89% for solid state and semi-solid state with 50, 75 and 100% water addition respectively. Statistical analysis showed that fermentation conditions had a significant effect ($p<0.05$) on the protein solubility of sorghum *Jand* prepared by using traditional and pure-culture of yeast and mold. Increasing water addition significantly increased protein solubility in both types of fermentation starters. LSD indicated that fermentation starter had a significant effect ($p<0.05$) on the protein solubility under the same fermentation condition. However, there was no significant difference in protein solubility in solid and semi-solid states with 50% water addition using traditional *murcha*.

Amongst the functional properties of proteins, solubility is probably the most critical because it affects other properties such as emulsification, foaming and gelation (Kinsella, 1976). Protein solubility characteristics are influenced by factors such as origin, processing conditions, pH, ionic strength and the presence of other ingredients (Kinsella, 1976). All samples have minimum protein solubility at pH 4, which is the isoelectric pH and the isoelectric pH of sorghum protein is 4.6 (Khattab *et al.*, 1972). Protein solubility of *Jand* samples prepared from traditional *murcha* is lower than that of pure-cultured starter. This can be explained in terms of pH, as it is one of the factor which plays great role in protein solubility. pH of *Jand* from traditional *murcha* had values more nearly concentrated towards isoelectric point and pH of *Jand* from traditional *murcha* was higher than that of pure culture, which may have caused more acidic protease to be active in the latter.

This may have led to an increase in the protein solubility resulting from hydrolysis of the storage proteins. Elmoneim *et al.* (2010) showed in their report that the highest solubility of the protein for germinated samples occurred at pH 6 and the values determined were 46.24%, 65.49%, 80.79%, 90.69%, 86.55% and 88.49% for the control, 1st, 2nd, 3rd, 4th and 5th day germinated sorghum flour, respectively. These values are far higher than that of protein solubility of sorghum (changed due to fermentation in this study). But, protein

solubility of sorghum was increased upto 58.06% in maximum after fermentation. When unmalted sample of sorghum *Jand* get fermented (12 days at 28ºC) after cooking (100ºC for 45 mins.), it results in significant increase in protein solubility and can be compared to the solubility of the seed after 24 hrs. of germination.

The values of protein solubility varied differently for pure culture and traditional *murcha*. Fermentation of sorghum by traditional *murcha* showed that there is no any significant difference ($p>0.05$) between solid state and semi-solid state with 50% water addition. This result gave promising scope of semi-solid state fermentation to the solid state, when traditional cultures are used, until protein solubility and sensory quality of sorghum *Jand* is in concern together. However, the result showed that protein solubility increased than the raw sorghum grain. The result showed that there is significant difference ($p<0.05$) between solid and all forms of semi-solid states, in tems of protein solubility, when used pure culture.

Sorghum proteins are divided into five major groups namely: albumins (soluble in water), globulins (soluble in salt), prolamins (soluble in 70-80% ethanol), glutelins (soluble in sodium hydroxide) and scleroproteins (insoluble in aqueous solvents); among which albumins are soluble in water. Yousif and Magboul (1972) studied the effect of fermentation on sorghum protein fractions and found that the albumin fraction increased significantly during the first 8 hours of fermentation. The protein solubility increased with increase in water content of semi-solid state. This may be due to the reason of well-growth of bacteria and mold in high water activity and corresponding production of protease enzyme. Low pH added into the production as well.

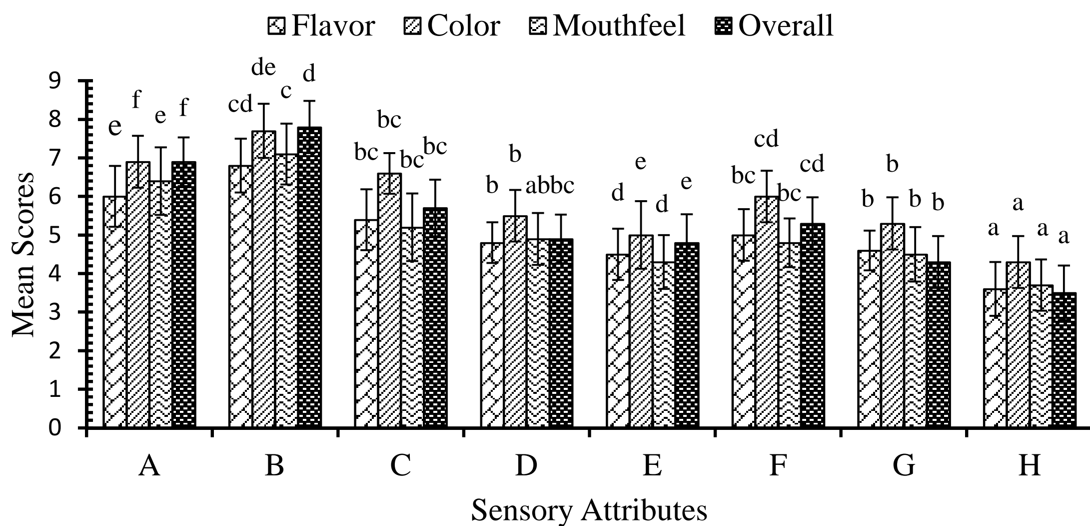
In semi-solid state fermentations, the insoluble solid substrate is a solid porous matrix, which absorbs water with a relatively high water activity and also contains available carbohydrates, nitrogen sources and mineral nutrients (Messens and Vuyst, 2002). And, reduction in pH during fermentation plays an important role in enhancing native proteolytic enzymes activity and consequently promotes the breakdown of proteins to smaller polypeptides which are easily digested by enzymes (Hamza, 1999). The semi-solid state fermentation process has great potential for enzyme production, and is of interest because raw fermented solid can be used as a substrate.

4.4 Sensory evaluation of sorghum *Jand*

Sensory evaluation of *Jand* was carried out by using 1 to 9 hedonic scales. Before sensory evaluation instructions panelists were instructed to give maximum scores for the samples coded by A, B, C, D, E, F, G, H which represented solid state fermentation by traditional *murcha* , solid state fermentation by pure culture, semi-solid state fermentation with 50% water added by pure culture, semi-solid state fermentation with 75% water added by pure culture, semi-solid state fermentation with 100% water added by pure culture, semi-solid state fermentation with 50% water added by traditional *murcha* , semi-solid state fermentation with 75% water added by traditional *murcha* and semi-solid state fermentation with 100% water added by traditional *murcha.*

10 semi-trained panelists were selected and there was no significant difference ($p>0.05$) between panelists over all sensory parameters suggesting that they have similarly judged the sensory parameters of the given 8 samples of sorghum *Jand.*

The statistical analysis of the sensory evaluation is shown in the appendix B and C. Bars with same letters for any sensory parameter are not significantly different at $p<0.05$.



*Bars having similar letter are not significantly different by LSD at p=0.05

Fig. 4.12 Sensory evaluation of sorghum *Jand*

4.4.1 Color

The *Jands* prepared from the sorghum as the substrate developed characteristics yellow-white color during fermentation. The tinge of yellowish color was greater in sorghum *Jand* when compared to that of finger millet *Jand.* The representation of codes A to H has been indicated earlier. The mean scores of color given by panelists were 6.9, 7.7, 6.6, 5.5, 5, 6, 5.3 and 4.3 for samples A, B, C, D, E, F, G and H respectively.

Mean scores for color were higher for both the solid states than each of the semi-solid states of fermentation; but mean score for *Jand* prepared from pure culture starter was higher than that of traditional *murcha* and both were significant different from each other ($p<0.05$) according to statistical analysis. Since, the value of protein solubility was higher in *jand* prepared from pure culture in solid state than the traditional *murcha* in the same state, color score was expected to be higher in respective way. This implied the result to be positive.

Among the 8 samples, statistical analysis showed that there was no significant difference ($p>0.05$) in color between samples A & C, C & D, C & F, D & E, D & F, D & G and E & G. Besides them, all the duet samples were significantly different from each other at $p<0.05$. Samples B and H i.e. solid state fermentation using pure culture and semi-solid state fermentation with 100 % water added using traditional *murcha* were significant different from every other samples in tems of color. The result also showed that sample coded D i.e. *Jand* from semi-solid state made with 75% added water and using pure culture resembled most of the other samples in semi-solid state by color at $p >0.05$.

Among the similarities, it was seen that samples A and C i.e. solid state fermentation by traditional *murcha* was not significantly different from semi-solid state fermentation with 50% water added by pure culture at $p>0.05$ in terms of color. No remarkable improvement on the sensory quality of color was seen using pure cultures or traditional *murcha,* when sorghum was fermented using semi-solid state, against solid state fermentation.

4.4.2 Flavor

Flavor is a perception that depends on the threshold level of the congeneric and acuity of panelist. Alcohols, esters, aldehydes, ketones, sulphur-containing compounds and organic acids are responsible for the organoleptic properties of alcoholic beverages (Batra and

Millner, 1974). Since alcohol content was not significantly different between solid and semi-solid state of fermentation, esters and aldehyde contents had supposed to effect flavor of the *Jand.* There were increasing ester content and decreasing aldehyde content from solid to semi-solid state with every stage of water addition, supported by increament in acidity to significantly higher level. Hence, more addition of water in semi-solid state fermentation, less acceptability in terms of flavor was expected. Also, since heterofermentative LAB are also important for the flavor because they produce the acids other than lactic acid; and since well-balanced ratio of lactic acid with other acid only gives the best flavor (Pederson, 1971), solid state of fermentation with pure culture was expected to secure highest score in terms of flavor.

The mean scores to the flavor were 6, 6.8, 5.4, 4.8, 4.5, 5, 4.6 and 3.6 for samples A, B, C, D, E, F, G and H. The statistical data at 5% level of significance showed that samples A & C, C & D, C & F, D & E, D & F, D & G, E & F, E & G and F & G are not significant different and all other duet samples were significant different to each other in terms of flavor. Samples B and H i.e. solid state fermentation using pure culture and semi-solid state fermentation with 100 % water added using traditional *murcha* respectively were significant different from every other samples in tems of flavor.

Among the similarities, it was seen that samples A and C i.e. solid state fermentation by traditional *murcha* was not significantly different from semi-solid state fermentation with 50% water added by pure culture at $p>0.05$ in terms of color. No remarkable improvement on the sensory quality of flavor was seen using pure cultures or traditional *murcha,* when sorghum was fermented using semi-solid state, against solid state fermentation.

4.4.3 Mouthfeel

In alcoholic beverages, mouth feel has to do mostly with the body (gravity), which depends on the degree of attenuation. It seems that alcohol and esters content may improve the mouthfeel of alcoholic beverages mostly (Berry and Chamberian, 1986). The alcohol contents were not significantly different within solid and semi-solid state fermentation, but it was significantly different amongst the use of traditional *murcha* and pure culture. So, *Jand* from pure culture was expected to secure higher scores. Esters content plays a vital role in mouthfeel of alcoholic beverages (Greiger and Piendl, 1976) and ester contents were significantly increasing with water addition in semi-solid state fermentation. But, too

much level of esters will also impair sensory quality of product and their values were high in the result of semi-solid states with increasing water content. So, overally mouthfeel of the sample with solid state fermentation by pure culture was expected to get highest score.

The mean scores to the mouthfeel were 6.4, 7.1, 5.2, 4.9, 4.3, 4.8, 4.5 and 3.7 for samples A, B, C, D, E, F, G and H. The statistical data at 5% level of significance showed that samples C & D, C & F, D & E, D & F, D & G, E & F, E & G, E & H and F & G are not significant different and all other duet samples were significant different to each other in terms of mouthfeel. Samples B and A i.e. solid state fermentation using pure culture and solid state fermentation using traditional *murcha* respectively were significant different from every other samples in tems of flavor.

Among the differences, unlike in color and flavor, it was seen that samples A and C i.e. solid state fermentation by traditional *murcha* was significantly different from semi-solid state fermentation with 50% water added by pure culture at $p<0.05$ in terms of mouthfeel. No remarkable improvement on the sensory quality of mouthfeel was seen using pure cultures or traditional *murcha,* when sorghum was fermented using semi-solid state, against solid state fermentation.

4.4.4 Overall acceptance

Color, flavor and mouthfeel have a combined effect on the overall acceptance of *Jand. Jand* prepared using pure culture in solid state had significantly superior flavor, color and mouthfeel at $p<0.05$. Hence, the overall acceptance of the sample was expected to secure highest score. And, since, color, flavor and mouthfeel for semi-solid state fermentation with increasing water content were given consecutively lower score; their acceptance was least expected.

The mean scores to the overall acceptance were 6.9, 7.8, 5.7, 4.9, 4.8, 5.3, 4.3 and 3.5 for samples A, B, C, D, E, F, G and H. The statistical data at 5% level of significance showed that samples C & F, D & E, D & F, D & G, E & F and E & G are not significant different and all other duet samples were significant different to each other in terms of overall acceptance. Samples B, Aand H i.e. solid state fermentation using pure culture, solid state fermentation using traditional *murcha* and semi-solid state fermentation using traditional

murcha respectively were significant different from every other samples in tems of overall acceptance.

Among the differences, unlike in color and flavor, it was seen that samples A and C i.e. solid state fermentation by traditional *murcha* was significantly different from semi-solid state fermentation with 50% water added by pure culture at p<0.05 in terms of overall acceptance. No remarkable improvement on the overall acceptance was seen using pure cultures or traditional *murcha,* when sorghum was fermented using semi-solid state, against solid state fermentation.

Part V

Conclusions and recommendations

5.1 Conclusions

The present work is still far too fragmentary. Because of severe time constraint as well as tedious nature of the work, only two *murcha* samples were taken for the study. Therefore conclusions drawn from this study may not be generally applicable for all *murcha* types. However, within the scope of the present work following general conclusions can be drawn.

- Enrichment step for encouraging the growth of fermentative yeasts in *murcha* samples is helpful in selecting only the hardiest (and therefore useful) yeasts and discouraging the trivial yeasts and other organisms.
- Starters of significantly superior sensory quality can be readily prepared from rice flour by using pure cultures of yeasts (propagated in molasses broth) and molds (propagated as *koji* in wheat bran). The preparation does not require any specialized tools and techniques and can be easily carried out at home level. Sanitation and hygiene, however, is essential.
- The type of *murcha* (the microbial profile of *murcha*, to be specific) significantly affects the sensory quality of *jand*. Although the relation of *murcha* and *jand* quality is quite intricate, alcohol content and taste have a significant bearing on the overall sensory quality of *jand*.
- Using mixed cultures (multistrain) does not necessarily produce good quality *murcha*. In fact, the synergistic effects of one organism type on the other must also be taken into account. The improvement of the microbiological quality of *murcha*, which means use of pure culture and exclusion of unwanted strains, the quality of finished product (both chemical and sensory) can be improved.
- Except moisture and alcohol contents, semi-solid state fermentations had a significant effect on the chemical and organoleptic qualities of sorghum *Jand* with substantial increase in total acidity, ester contents and protein solubility of sorghum proteins; and substantial decrease in pH, aldehyde contents, methanol and reducing sugar.
- Except moisture content, pure cultures had a significant effect on the chemical and organoleptic qualities of sorghum *Jand* with substantial increase in total acidity,

alcohol by volume, ester contents and protein solubility of sorghum proteins; and substantial decrease in pH, aldehyde contents, methanol and reducing sugar.

- From sensory point of view, regarding color and flavor; Semi-solid fermentation with 50% water addition using pure culture resembled solid state fermentation using traditional *murcha.*
- Color, flavor, mouthfeel and overall acceptance were largely impaired by semi-solid fermentation with profound impact impact on flavor. No remarkable improvement on the overall quality of the sorghum *Jand* was found using semi-solid fermentation over solid state one.

5.2 Recommendations

Based on the present study, following recommendations for further study and home-scale trials on *murcha* making can be made.

- All possible types of essential organisms need to be screened, characterized, and their role in the fermentation elucidated. This, however, can be challenging, particularly in terms of time and budget.
- There is plenty of scope for similar study on starter preparation using *murcha* plants (rather than *murcha*) as the source of essential organisms (fermenting yeasts, saccharifying molds).
- Reliable objective techniques need to be developed for quantifying the shelf-life and evaluating quality of *murcha* as well as sorghum *Jand.*
- Optimization of fermentation parameters like temperature and pH can be studied.
- Detail study about the enzymatic activity of pure culture can be studied.
- Detail study on effects of cooking and fermentation on protein digestibility and solubility of sorghum can be studied.
- Effects of anti-nutrients on sensory and chemical properties of *Jand* can be studied. Red species of sorghum can be taken for this study which contains high tannins.
- Substantial decrement of reducing sugar from solid to semi-solid state fermentation can be studied in detail about the cause and optimization if possible.

Part VI

Summary

Alcoholic beverages prepared from *murcha* are popular in Nepal and large number of people consumes it. There is no fixed data about the production of *murcha* and consumption of beverages made from it. Directly or indirectly, health of large number of people is associated with it. Compromise with the production of *murcha* is no way a good practice, but lack of proper knowledge and carelessness has made us to do so. This work deals with the problem of *murcha* making which not only solves issues regarding health but also improves the quality of product made from it. Besides this, there is lack of relevant informations about semi-solid state fermentation. Most of the fermentation process undergo by solid substrate and submerged state fermentation in commercial scale. In current days research and investigations are being held regarding semi-solid state fermentation, especially in the production of bio-fuels, as it has great potential for enzyme production, and is of interest because raw fermented solid can be used as a substrate. This work, therefore, helps to find the scope of semi-solid state fermentation on food and beverages other than bio-fuels.

With the hypothesis that starters prepared from pure cultures give better quality, attempt was made to prepare starter cake in the laboratory using pure cultures isolated from local *murcha* collected from street of Dharan in Nepal. The initial screening of fermentative yeasts and saccharifying molds was carried out using modification of the methods described by different authors. The isolates were found to consist of single-type yeast (*Saccharomyces* spp.) and mold (*Rhizopus* spp) cultures. The cultures could be easily maintained for up to 6 months by standard subculturing process in MYGP agar. Mold could also be propagated as *koji* in sterilized (autoclaved) wheat bran by inoculating the latter with pure mold spores followed by incubation in moist chamber at 28-30ºC and 95% RH for 5-6 days. The *koji* (dried spontaneously to ~ 14% moisture content) showed an excellent stability at room temperature (~ 30ºC) and was subsequently used as for starter preparation. Starter cakes (dia ~ 2cm, thickness ~ 0.5 cm) were prepared in moist rice flour (~ 50% moisture content) by mixing 0.5% (m/m) each of pure cultures of yeast and mold (isolated from traditional *murcha*). Yeast was used in the form of sediment (collected after culturing in 5% molasses broth for 3 days at 30ºC). Incubation of starter cakes at 28-30ºC

and 95% RH for 2 days gave the best result (with highly characteristic appearance). The cakes were subjected to two-stage drying at < 55ºC in cabinet dryer to bring down the moisture to ~ 16%.

Fermentation of sorghum was done by using market *murcha* (0.5%w/w) and pure culture (0.5%w/w) for 12 days (as per highest acidity value) at 28ºC after cooking for 45 mins. (completion of gelation of starch) at 100ºC (below denaturation point of sorghum protein to evaluate exact change in protein solubility by fermentation). Fermentation was carried out in solid and semi-solid states with 50%, 75% and 100% water addition (in order to show effect of the state with constant fold of water addition). Use of pure culture gave significantly higher value of alcohol content compared to that of market *murcha* in all fermentation state but there was no significant difference in alcohol content within solid and semi-solid state fermentation for both type of *murchas.* Moisture contents were not found significantly different when sorghum got fermented by using any of the *murchas* and in any of the fermentation states. There was no significant difference in aldehyde contents between solid and semi-solid (50%) states and between semi-solid states 75% and 100% water addition. Reducing sugar drastically decreased from solid to semi-solid state fermentation and remained no significant different within all given semi-solid states. Other chemical parameters (pH, esters content, methanol and acidity) were found significantly different in all fermentation states and between both traditional *murcha* and pure culture. The significant differences were carried out at 5% level of significance.

Above all, most important finding of this study was to show change in protein solubility of sorghum proteins; and the values increased than raw sorghum when got fermented by pure cultures. Protein solubility of sorghum protein got increased significantly, when fermented by semi-solid state and the value increased with the increment in proportion of water addition. Result also showed that there was no any significant difference between solid and semi-solid (50%) states in terms of protein solubility when traditional *murcha* was used and there was also no significant difference between them in terms of color and flavor; but there was significant difference in mouthfeel and overall acceptance between them. This demands further research on the acceptability of semi-solid state fermentation of sorghum upto threshold of 50% water addition, when protein solubility is in major concern.

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Appendices

Appendix A

Specimen card for sensory evaluation

Hedonic rating test

Name of panelist: **Date:**

Product: Sorghum *Jand*

Please taste these given products and check how much you like or dislike each one i.e. by your perception of individual parameters. Please, give points for your like or dislike as given below, for each parameters.

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

Sample / Parameter	A	B	C	D	E	F	G	H
Color								
Flavor								
Mouth feel								
Overall Acceptance								

Comments:………………………………………………………………………………………

…………………………………………………………………………………………………

Signature

Source: (Ranganna, 2005)

Appendix B

ANOVA results for sensory analysis

Table B.1 Two way ANOVA (no blocking) for variate flavor

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Panelist	9	3.5125	0.3903	0.84	0.585
Sample	7	67.4875	9.6411	20.67	<.001
Residual	63	29.3875	0.4665		
Total	79	100.3875			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table B.1.1 LSD for flavor

LSD at 0.05=0.6104

Sample Code	Mean Score	Mean difference			
A	6.0	A-B>LSD*	B-D> LSD*	C-G> LSD*	E-H> LSD*
B	6.8	A-C<LSD	B-E> LSD*	C-H> LSD*	F-G<LSD
C	5.4	A-D> LSD*	B-F> LSD*	D-E<LSD	F-H> LSD*
D	4.8	A-E> LSD*	B-G> LSD*	D-F<LSD	G-H> LSD*
E	4.5	A-F> LSD*	B-H> LSD*	D-G<LSD	
F	5.0	A-G> LSD*	C-D<LSD	D-H> LSD*	
G	4.6	A-H> LSD*	C-E> LSD*	E-F<LSD	
H	3.6	B-C> LSD*	C-F<LSD	E-G<LSD	

*= significantly different

Table B.2 Two way ANOVA (no blocking) for variate color

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Panelist	9	2.5125	0.2792	0.56	0.827
Sample	7	86.2875	12.3268	24.59	<.001
Residual	63	31.5875	0.5014		
Total	79	120.3875			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table B.2.1 LSD for color

LSD at 0.05=0.6328

Sample Code	Mean Score	Mean difference			
A	6.9	A-B>LSD*	B-D> LSD*	C-G> LSD*	E-H> LSD*
B	7.7	A-C<LSD	B-E> LSD*	C-H> LSD*	F-G>LSD*
C	6.6	A-D> LSD*	B-F> LSD*	D-E<LSD	F-H> LSD*
D	5.5	A-E> LSD*	B-G> LSD*	D-F<LSD	G-H> LSD*
E	5.0	A-F> LSD*	B-H> LSD*	D-G<LSD	
F	6.0	A-G> LSD*	C-D<LSD	D-H> LSD*	
G	5.3	A-H> LSD*	C-E> LSD*	E-F>LSD*	
H	4.3	B-C> LSD*	C-F<LSD	E-G<LSD	

*= significantly different

Table B.3 Two way ANOVA (no blocking) for variate mouthfeel

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Panelist	9	4.6125	0.5125	0.91	0.523
Sample	7	87.8875	12.5554	22.29	<.001
Residual	63	35.4875	0.5633		
Total	79	127.9875			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table B.3.1 LSD for mouthfeel

LSD at 0.05=0.6707

Sample Code	Mean Score	Mean difference			
A	6.4	A-B>LSD*	B-D> LSD*	C-G> LSD*	E-H< LSD
B	7.1	A-C>*LSD	B-E> LSD*	C-H> LSD*	F-G<LSD
C	5.2	A-D> LSD*	B-F> LSD*	D-E<LSD	F-H> LSD*
D	4.9	A-E> LSD*	B-G> LSD*	D-F<LSD	G-H> LSD*
E	4.3	A-F> LSD*	B-H> LSD*	D-G<LSD	
F	4.8	A-G> LSD*	C-D<LSD	D-H> LSD*	
G	4.5	A-H> LSD*	C-E> LSD*	E-F<LSD	
H	3.7	B-C> LSD*	C-F<LSD	E-G<LSD	

*= significantly different

Table B.4 Two way ANOVA (no blocking) for variate overall

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Panelist	9	2.7	0.3	0.61	0.786
Sample	7	135.4	19.3429	39.18	<.001
Residual	63	31.1	0.4937		
Total	79	169.2			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table B.4.1 LSD for overall

LSD at 0.05=0.6279

Sample Code	Mean Score	Mean difference			
A	6.9	A-B>LSD*	B-D> LSD*	C-G> LSD*	E-H> LSD*
B	7.8	A-C>LSD*	B-E> LSD*	C-H> LSD*	F-G>LSD*
C	5.7	A-D> LSD*	B-F> LSD*	D-E<LSD	F-H> LSD*
D	4.9	A-E> LSD*	B-G> LSD*	D-F<LSD	G-H> LSD*
E	4.8	A-F> LSD*	B-H> LSD*	D-G<LSD	
F	5.3	A-G> LSD*	C-D>LSD*	D-H> LSD*	
G	4.3	A-H> LSD*	C-E> LSD*	E-F<LSD	
H	3.5	B-C> LSD*	C-F<LSD	E-G<LSD	

*= significantly different

Appendix C

ANOVA results for chemical analysis on effect of fermentation state by any of the traditional or pure culture starter

C.1 Effect of traditional *murcha* on different fermentation state

Table C.1.1 One way ANOVA for variate fermentation state(*ferment*) and factor *acidity*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	1.153863	0.384621	242.68	<.001
Residual	8	0.012679	0.001585		
Total	11	1.166542			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.1.2 LSD for acidity

LSD at 0.05=0.075

Sample Code	Mean Score	Mean difference	
S	0.57	S-S1>LSD*	S1-S3> LSD*
S1	0.8727	S-S2>*LSD	S2-S3> LSD*
S2	1.1533	S-S3> LSD*	
S3	1.4	S1-S2> LSD*	

*= significantly different

Table C.1.3 One way ANOVA for variate fermentation state(*ferment*) and factor *alcohol*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	0.132	0.044	0.70	0.579
Residual	8	0.50487	0.06311		
Total	11	0.63687			

Since F pr > 0.05, there is no significantly difference between the samples so, LSD testing is not necessary.

Table C.1.4 LSD for alcohol

LSD at 0.05=0.473

Table C.1.5 One way ANOVA for variate fermentation state(*ferment*) and factor *aldehyde*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	2.68063	0.89354	19.87	<.001
Residual	8	0.35980	0.04497		
Total	11	3.04042			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.1.6 LSD for aldehyde

LSD at 0.05=0.3993

Sample Code	Mean Score	Mean difference	
S	3.190	S-S1<LSD	S1-S3> LSD*
S1	2.973	S-S2>*LSD	S2-S3< LSD
S2	2.323	S-S3> LSD*	
S3	2.023	S1-S2> LSD*	

*= significantly different

Table C.1.7 One way ANOVA for variate fermentation state(*ferment*) and factor *dry matter content*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	0.135	0.045	0.87	0.495
Residual	8	0.41347	0.05168		
Total	11	0.54847			

Since F pr > 0.05, there is no significantly difference between the samples so, LSD testing is not necessary.

Table C.1.8 LSD for dry matter content

LSD at 0.05=0.4280

*= significantly different

Table C.1.9 One way ANOVA for variate fermentation state(*ferment*) and factor *esters*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	113.2922	37.7641	70.16	<.001
Residual	8	4.3061	0.5383		
Total	11	117.5983			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.1.10 LSD for esters

LSD at 0.05=1.381

Sample Code	Mean Score	Mean difference	
S	13.53	S-S1>LSD*	S1-S3> LSD*
S1	15.40	S-S2>*LSD	S2-S3> LSD*
S2	17.24	S-S3> LSD*	
S3	21.81	S1-S2> LSD*	

*= significantly different

Table C.1.11 One way ANOVA for variate fermentation state(*ferment*) and factor methanol

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	0.00284565	0.00094855	82.11	<.001
Residual	8	0.00009242	0.00001155		
Total	11	0.00293907			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.1.12 LSD for methanol

LSD at 0.05=0.0064

Table C.13 One way ANOVA for variate fermentation state(*ferment*) and factor *pH*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	1.3929	0.4643	21.87	<.001
Residual	8	0.16987	0.02123		
Total	11	1.56277			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.1.14 LSD for pH (pure culture *murcha*)

LSD at 0.05=0.2744

Sample Code	Mean Score	Mean difference	
S	3.37	S-S1>LSD*	S1-S3> LSD*
S1	3.05	S-S2>*LSD	S2-S3> LSD*
S2	2.77	S-S3> LSD*	
S3	2.45	S1-S2> LSD*	

*= significantly different

Table C.1.15 One way ANOVA for variate fermentation state (*ferment*) and factor *protein solubility*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	55.9337	18.6446	24.04	<.001
Residual	8	6.2056	0.7757		
Total	11	62.1393			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.1.16 LSD for protein solubility (pure culture *murcha*)

LSD at 0.05=1.658

Sample Code	Mean Score	Mean difference	
S	39.05	S-S1>LSD*	S1-S3> LSD*
S1	41.26	S-S2>*LSD	S2-S3> LSD*
S2	43	S-S3> LSD*	
S3	44.89	S1-S2> LSD*	

*= significantly different

Table C.1.17 One way ANOVA for variate fermentation state(*ferment*) and factor *reducing sugar*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	0.537236	0.179079	67.80	<.001
Residual	8	0.021131	0.002641		
Total	11	0.558367			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.1.18 LSD for reducing sugar

LSD at 0.05=0.0968

Sample Code	Mean Score	Mean difference	
S	0.8583	S-S1>LSD*	S1-S3< LSD
S1	0.3963	S-S2>*LSD	S2-S3< LSD
S2	0.3703	S-S3> LSD*	
S3	0.3473	S1-S2< LSD	

*= significantly different

C.2 Effect of pure cultured *murcha* on different fermentation state

Table C.2.1 One way ANOVA for variate fermentation state(*ferment*) and factor *acidity*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	1.773459	0.591153	304.56	<.001
Residual	8	0.015528	0.001941		
Total	11	1.788987			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.2.2 LSD for acidity

LSD at 0.05=0.083

Sample Code	Mean Score	Mean difference	
S	0.825	S-S1>LSD*	S1-S3> LSD*
S1	0.975	S-S2>*LSD	S2-S3> LSD*
S2	1.607	S-S3> LSD*	
S3	1.710	S1-S2> LSD*	

*= significantly different

Table C.2.3 One way ANOVA for variate fermentation state(*ferment*) and factor *alcohol*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	0.46897	0.15632	3.33	0.077
Residual	8	0.37513	0.04689		
Total	11	0.84410			

Since F pr > 0.05, there is no significantly difference between the samples so, LSD testing is not necessary.

Table C.2.4 LSD for alcohol

LSD at 0.05=0.4077

Table C.2.5 One way ANOVA for variate fermentation state(*ferment*) and factor *aldehyde*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	2.07896	0.69299	15.09	0.001
Residual	8	0.36747	0.04593		
Total	11	2.44642			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.2.6 LSD for aldehyde

LSD at 0.05=0.4035

Sample Code	Mean Score	Mean difference	
S	2.503	S-S1<LSD	S1-S3> LSD*
S1	2.357	S-S2>*LSD	S2-S3< LSD
S2	1.827	S-S3> LSD*	
S3	1.463	S1-S2> LSD*	

*= significantly different

Table C.2.7 One way ANOVA for variate fermentation state(*ferment*) and factor *dry matter*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	0.4596	0.1532	1.39	0.314
Residual	8	0.8810	0.1101		
Total	11	1.3406			

Since F pr >0.05, there is no significantly difference between the samples so, LSD testing is not necessary.

Table C.2.8 LSD for dry matter

LSD at 0.05=0.6248

Table C.2.9 One way ANOVA for variate fermentation state(*ferment*) and factor *esters*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	167.7635	55.9212	124.75	<.001
Residual	8	3.5861	0.4483		
Total	11	171.3496			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.2.10 LSD for esters

LSD at 0.05=1.261

Sample Code	Mean Score	Mean difference	
S	15.44	S-S1>LSD*	S1-S3> LSD*
S1	17.66	S-S2>*LSD	S2-S3> LSD*
S2	20.17	S-S3> LSD*	
S3	25.48	S1-S2> LSD*	

*= significantly different

Table C.2.11 One way ANOVA for variate fermentation state(*ferment*) and factor *methanol*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	0.00259244	0.00086415	84.44	<.001
Residual	8	0.00008187	0.00001023		
Total	11	0.00267431			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.2.12 LSD for methanol

LSD at 0.05=0.006023

Sample Code	Mean Score	Mean difference	
S	0.04913	S-S1>LSD*	S1-S3> LSD*
S1	0.02067	S-S2>*LSD	S2-S3> LSD*
S2	0.02567	S-S3> LSD*	
S3	0.00867	S1-S2< LSD	

*= significantly different

Table C.2.13 One way ANOVA for variate fermentation state(*ferment*) and factor *pH*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	1.61390	0.53797	26.39	<.001
Residual	8	0.16307	0.02038		
Total	11	1.77697			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.2.14 LSD for pH (traditional *murcha*)

LSD at 0.05=0.2688

Sample Code	Mean Score	Mean difference	
S	4.01	S-S1>LSD*	S1-S3> LSD*
S1	3.69	S-S2>LSD*	S2-S3> LSD*
S2	3.36	S-S3> LSD*	
S3	3.02	S1-S2> LSD*	

*= significantly different

Table C.2.15 One way ANOVA for variate fermentation state (*ferment*) and factor *protein solubility*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	64.721	21.574	13.90	0.002
Residual	8	12.415	1.552		
Total	11	77.135			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.2.16 LSD for protein solubility

LSD at 0.05=2.346 (traditional *murcha*)

Sample Code	Mean Score	Mean difference	
S	32.5	S-S1<LSD	S1-S3> LSD*
S1	32.78	S-S2>*LSD	S2-S3<LSD
S2	36.01	S-S3> LSD*	
S3	38.08	S1-S2> LSD*	

*= significantly different

Table C.2.17 One way ANOVA for variate fermentation state(*ferment*) and factor *reducing sugar*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Ferment	3	0.265427	0.088476	30.50	<.001
Residual	8	0.023205	0.002901		
Total	11	0.288632			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table C.2.18 LSD for reducing sugar

LSD at 0.05=0.1014

Sample Code	Mean Score	Mean difference	
S	0.589	S-S1>LSD*	S1-S3< LSD
S1	0.277	S-S2>*LSD	S2-S3< LSD
S2	0.249	S-S3> LSD*	
S3	0.220	S1-S2< LSD	

*= significantly different

Appendix D

D.1 Effect of traditional and pure-cultured *murcha* on solid state fermentation

Table D.1.1 One way ANOVA for variate *murcha* and factor *acidity*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.0977927	0.0977927	187.10	<.001
Residual	4	0.0020907	0.0005227		
Total	5	0.0998833			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.1.2 LSD for acidity

LSD at 0.05=0.05183

Table D.1.3 One way ANOVA for variate *murcha* and factor *alcohol*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	1.58107	1.58107	58.41	0.002
Residual	4	0.10827	0.02707		
Total	5	1.68933			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.1.4 LSD for alcohol

LSD at 0.05=0.3729

Table D.1.5 One way ANOVA for variate *murcha* and factor *aldehyde*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.70727	0.70727	12.83	0.023
Residual	4	0.22047	0.05512		
Total	5	0.92773			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.1.6 LSD for aldehyde

LSD at 0.05=0.5322

Table D.1.7 One way ANOVA for variate *murcha* and factor *dry matter*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.13500	0.13500	2.94	0.162
Residual	4	0.18393	0.04598		
Total	5	0.31893			

Since F pr >0.05, there is no significantly difference between the samples.

Table D.1.8 LSD for dry matter

LSD at 0.05= 0.4861

Table D.1.9 One way ANOVA for variate *murcha* and factor *esters*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	5.4722	5.4722	10.07	0.034
Residual	4	2.1728	0.5432		
Total	5	7.6450			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.1.10 LSD for esters

LSD at 0.05=1.671

Table D.1.11 One way ANOVA for variate *murcha* and factor *methanol*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	3.466E-04	3.466E-04	40.07	0.003
Residual	4	3.459E-05	8.648E-06		
Total	5	3.812E-04			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.1.12 LSD for methanol

LSD at 0.05= 0.00667

Table D.1.13 One way ANOVA for variate *murcha* and factor *pH*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.60167	0.60167	21.97	0.009
Residual	4	0.10953	0.02738		
Total	5	0.71120			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.1.14 LSD for pH

LSD at 0.05= 0.3751

Table D.1.15 One way ANOVA for variate *murcha* and factor *protein solubility*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	64.288	64.288	41.58	0.003
Residual	4	6.185	1.546		
Total	5	70.473			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.1.16 LSD for protein solubility

LSD at 0.05= 2.819

Table D.1.17 One way ANOVA for variate *murcha* and factor *reducing sugar*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.108811	0.108811	13.40	0.022
Residual	4	0.032471	0.008118		
Total	5	0.141281			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.1.18 LSD for reducing sugar

LSD at 0.05= 0.2042

D.2 Effect of traditional and pure-cultured *murcha* on semi-solid state fermentation with 50 %(v/m) added

Table D.2.1 One way ANOVA for variate *murcha* and factor *acidity*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.0158107	0.0158107	39.94	0.003
Residual	4	0.0015833	0.0003958		
Total	5	0.0173940			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.2.2 LSD for acidity

LSD at 0.05= 0.04510

Table D.2.3 One way ANOVA for variate *murcha* and factor *alcohol*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	1.19707	1.19707	30.99	0.005
Residual	4	0.15453	0.03863		
Total	5	1.35160			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.2.4 LSD for alcohol

LSD at 0.05= 0.4456

Table D.2.5 One way ANOVA for variate *murcha* and factor *aldehyde*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.57042	0.57042	8.12	0.046
Residual	4	0.28113	0.07028		
Total	5	0.85155			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.2.6 LSD for aldehyde

LSD at 0.05= 0.601

Table D.2.7 One way ANOVA for variate *murcha* and factor *dry matter*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.04507	0.04507	0.45	0.537
Residual	4	0.39627	0.09907		
Total	5	0.44133			

Since F pr >0.05, there is no significantly difference between the samples.

Table D.2.8 LSD for dry matter

LSD at 0.05= 0.4861

Table D.2.9 One way ANOVA for variate *murcha* and factor *esters*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	7.6840	7.6840	41.91	0.003
Residual	4	0.7335	0.1834		
Total	5	8.4175			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.2.10 LSD for esters

LSD at 0.05= 0.971

Table D.2.11 One way ANOVA for variate *murcha* and factor *methanol*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.00093750	0.00093750	63.27	0.001
Residual	4	0.00005927	0.00001482		
Total	5	0.00099677			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.2.12 LSD for methanol

LSD at 0.05= 0.00873

Table D.2.13 One way ANOVA for variate *murcha* and factor *pH*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.60167	0.60167	27.88	0.006
Residual	4	0.08633	0.02158		
Total	5	0.68800			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.2.14 LSD for pH

LSD at 0.05= 0.3330

Table D.2.15 One way ANOVA for variate *murcha* and factor *protein solubility*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	107.9504	107.9504	113.80	<.001
Residual	4	3.7943	0.9486		
Total	5	111.7447			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.2.16 LSD for protein solubility

LSD at 0.05= 2.208

Table D.2.17 One way ANOVA for variate *murcha* and factor *reducing sugar*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.021361	0.021361	12.49	0.024
Residual	4	0.006839	0.001710		
Total	5	0.028199			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.2.18 LSD for reducing sugar

LSD at 0.05= 0.0937

D.3 Effect of traditional and pure-cultured *murcha* on semi-solid state fermentation with 75 %(v/m) added

Table D.3.1 One way ANOVA for variate *murcha* and factor *acidity*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.308267	0.308267	105.09	<.001
Residual	4	0.011733	0.002933		
Total	5	0.320000			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.3.2 LSD for acidity

LSD at 0.05= 0.1228

Table D.3.3 One way ANOVA for variate *murcha* and factor *alcohol*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.63375	0.63375	14.46	0.019
Residual	4	0.17533	0.04383		
Total	5	0.80908			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.3.4 LSD for alcohol

LSD at 0.05= 0.4746

Table D.3.5 One way ANOVA for variate *murcha* and factor *aldehyde*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.37002	0.37002	12.72	0.023
Residual	4	0.11633	0.02908		
Total	5	0.48635			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is

necessary.

Table D.3.6 LSD for aldehyde

LSD at 0.05= 0.3866

Table D.3.7 One way ANOVA for variate *murcha* and factor *dry matter*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.0088	0.0088	0.08	0.789
Residual	4	0.4293	0.1073		
Total	5	0.4381			

Since F pr >0.05, there is no significantly difference between the samples.

Table D.3.8 LSD for dry matter

LSD at 0.05= 0.743

Table D.3.9 One way ANOVA for variate *murcha* and factor *esters*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	12.8188	12.8188	22.04	0.009
Residual	4	2.3265	0.5816		
Total	5	15.1453			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.3.10 LSD for esters

LSD at 0.05 =1.729

Table D.3.11 One way ANOVA for variate *murcha* and factor *methanol*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.00025091	0.00025091	15.77	0.017
Residual	4	0.00006363	0.00001591		
Total	5	0.00031453			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.3.12 LSD for methanol

LSD at 0.05= 0.00904

Table D.3.13 One way ANOVA for variate *murcha* and factor *pH*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.52215	0.52215	23.81	0.008
Residual	4	0.08773	0.02193		
Total	5	0.60988			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.3.14 LSD for pH

LSD at 0.05= 0.3357

Table D.3.15 One way ANOVA for variate *murcha* and factor *protein solubility*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	73.2901	73.2901	94.85	<.001
Residual	4	3.0908	0.7727		
Total	5	76.3809			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.3.16 LSD for protein solubility

LSD at 0.05= 1.993

Table D.3.17 One way ANOVA for variate *murcha* and factor *reducing sugar*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.0222042	0.0222042	33.73	0.004
Residual	4	0.0026333	0.0006583		
Total	5	0.0248375			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.3.18 LSD for reducing sugar

LSD at 0.05=0.0582

D.4 Effect of traditional and pure-cultured *murcha* on semi-solid state fermentation with 100 %(v/m) added

Table D.4.1 One way ANOVA for variate *murcha* and factor *acidity*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.144150	0.144150	45.05	0.003
Residual	4	0.012800	0.003200		
Total	5	0.156950			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.4.2 LSD for acidity

LSD at 0.05= 0.1282

Table D.4.3 One way ANOVA for variate *murcha* and factor *alcohol*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.9520	0.9520	8.62	0.043
Residual	4	0.4419	0.1105		
Total	5	1.3939			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.4.4 LSD for alcohol

LSD at 0.05= 0.753

Table D.4.5 One way ANOVA for variate *murcha* and factor *aldehyde*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.47040	0.47040	17.21	0.014
Residual	4	0.10933	0.02733		
Total	5	0.57973			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.4.6 LSD for aldehyde

LSD at 0.05= 0.3748

Table D.4.7 One way ANOVA for variate *murcha* and factor *dry matter*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.00735	0.00735	0.10	0.764
Residual	4	0.28493	0.07123		
Total	5	0.29228			

Since F pr >0.05, there is no significantly difference between the samples.

Table D.4.8 LSD for dry matter

LSD at 0.05= 0.605

Table D.4.9 One way ANOVA for variate *murcha* and factor *esters*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	20.2401	20.2401	30.44	0.005
Residual	4	2.6595	0.6649		
Total	5	22.8995			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.4.10 LSD for esters

LSD at 0.05 =1.848

Table D.4.11 One way ANOVA for variate *murcha* and factor *methanol*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	2.419E-04	2.419E-04	57.63	0.002
Residual	4	1.679E-05	4.198E-06		
Total	5	2.587E-04			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.4.12 LSD for methanol

LSD at 0.05= 0.004645

Table D.4.13 One way ANOVA for variate *murcha* and factor *pH*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.48735	0.48735	39.51	0.003
Residual	4	0.04933	0.01233		
Total	5	0.53668			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.4.14 LSD for pH

LSD at 0.05= 0.2517

Table D.4.15 One way ANOVA for variate *murcha* and factor *protein solubility*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	70.658	70.658	53.29	0.002
Residual	4	5.304	1.326		
Total	5	75.962			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.4.16 LSD for protein solubility

LSD at 0.05= 2.610

Table D.4.17 One way ANOVA for variate *murcha* and factor *reducing sugar*

Source of variation	d.f.	s.s.	m.s.	v.r.	F.pr.
Murcha	1	0.0241935	0.0241935	40.43	0.003
Residual	4	0.0023933	0.0005983		
Total	5	0.0265868			

Since F pr < 0.05, there is significantly difference between the samples so, LSD testing is necessary.

Table D.4.18 LSD for reducing sugar

LSD at 0.05= 0.05545

Appendix E

Keys to differentiation of common yeast genera and species

1. Vegetative reproduction by cross-wall formation followed by fission – Schizosaccharomyces

1. Vegetative reproduction by budding – 2

2. Ascospores not formed – ***Candida***

2. Ascospores formed – 3

3. Nitrate assimilated – ***Hansenula***

3. Nitrate not assimilated – 4

4. Abundant true mycelium as well as budding – 5

4. True mycelium scarce or absent – 6

5. Asci formed exclusively on the true hyphae – ***Saccharomycopsis***

5. Asci not formed exclusively on the true hyphae – ***Pichia***

6. Asci dehiscent – ***Kluyveromyces***

6. Asci persistent – 7

7. No conjugation preceding ascus formation – ***Saccharomyces***

7. Conjugation preceding ascus formation – 8

8. Ascospores warty or with ridges – ***Debaryomyces***

8. Ascospores spherical and smooth – ***Zygosaccharomyces***

The classical methods for the differentiation of species entail sugar assimilation, cycloheximide resistance, fermentation, nitrogen utilization, urea hydrolysis, and temperature studies. An example of how ***Saccharomyces cerevisiae*** may be differentiated is given as follows (Harrigan and McCance, 1976):

- Glucose is always strongly fermented
- No true mycelium but a pseudomycelium and/or loose collection of budding cells may be formed
- Multilateral budding
- Variable dependence on ethanol as the sole source of carbon
- Sensitive to actidione

Responses to (assimilation) sugars are as follows:

- Glucose: positive
- Maltose: Mostly positive
- Galactose: Mostly positive
- Sucrose: positive
- Lactose: negative
- Fructose: positive

Saccharomyces uvarum and ***Hansenula spp*** may show similar responses but the former cannot use ethanol as the sole source of carbon while the latter forms a dry, dull pellicle on malt broth.

Appendix F

Table F.1 Effects of solid and semi-solid state on the chemical characteristics of sorghum *Jand* using traditional *murcha*

Parameters	Values*			
	Solid state	Semi-solid (50%)	Semi-solid (75%)	Semi-solid (100%)
Total acidity (%m/v)	0.57^{a} (0.026)	0.873^{b} (0.018)	1.153^{c} (0.042)	1.4^{d} (0.06)
pH	4.01^{a} (0.19)	3.69^{b} (0.11)	3.36^{c} (0.14)	3.02^{d} (0.117)
Alcohol (%v/v)	4.133^{a} (0.155)	4.113^{a} (0.215)	4.093^{a} (0.186)	3.873^{a} (0.384)
Dry matter content (%)	4.137^{a} (0.185)	4.077^{a} (0.275)	3.947^{a} (0.225)	3.867^{a} (0.215)
Esters (g/100L of absolute alcohol)	13.53^{a} (0.605)	15.4^{b} (0.45)	17.24^{c} (0.82)	21.81^{d} (0.955)
Aldehydes (g/100L of absolute alcohol)	3.19^{a} (0.214)	2.973^{a} (0.276)	2.323^{b} (0.162)	2.023^{b} (0.179)
Methanol (%m/v)	0.0643^{a} (0.0021)	0.0457^{b} (0.005)	0.039^{c} (0.004)	0.021^{d} (0.0014)
Reducing sugar (%m/v)	0.858^{a} (0.096)	0.396^{b} (0.022)	0.37^{b} (0.026)	0.347^{b} (0.015)
Protein solubility (%m/m)	32.5^{a} (1.554)	32.78^{a} (1.1)	36.01^{b} (1.214)	38.08^{b} (1.053)

Table F.2 Effects of solid and semi-solid state on the chemical characteristics of sorghum *Jand* using pure culture

Parameters	Values*			
	Solid state	Semi-solid (50%)	Semi-solid (75%)	Semi-solid (100%)
Total acidity (%m/v)	0.825^{a} (0.019)	0.975^{b} (0.022)	1.607^{c} (0.064)	1.71^{d} (0.053)
pH	3.37^{a} (0.137)	3.05^{b} (0.176)	2.77^{c} (0.156)	2.45^{d} (0.105)
Alcohol (%v/v)	5.16^{a} (0.173)	5.007^{a} (0.176)	4.743^{a} (0.231)	4.67^{a} (0.271)
Dry matter content (%)	4.437^{a} (0.24)	4.25^{a} (0.35)	4.023^{a} (0.405)	3.937^{a} (0.31)
Esters (g/100L)	15.44^{a} (0.849)	17.66^{b} (0.405)	20.17^{c} (0.701)	25.48^{d} (0.646)
Aldehydes (g/100L)	2.503^{a} (0.019)	2.357^{a} (0.022)	1.827^{b} (0.064)	1.463^{b} (0.053)
Methanol (%m/v)	0.0491^{a} (0.0029)	0.0206^{b} (0.0031)	0.026^{b} (0.004)	0.0087^{c} (0.003)
Reducing sugar (%m/v)	0.589^{a} (0.084)	0.277^{b} (0.054)	0.249^{b} (0.025)	0.22^{b} (0.031)
Protein solubility (%)	39.05^{a} (0.821)	41.26^{b} (0.822)	43^{c} (0.259)	44.89^{d} (1.298)

*Means followed by different superscripts in a row are significantly different ($p<0.05$) by LSD. Figures in the parentheses are standard deviations. Semi-solid 50%, 75% and 100% are the semi-solid state fermentations using 50%, 75% and 100% water addition respectively to biomass developed sorghum during alcoholic fermentation.

Table F.3 L.S.D.(at 5% level of significance) table for the effects of fermentation states on different parameters of sorghum *Jand*

Fermentation starters	Traditional murcha	Pure-culture
Parameters		
Acidity	0.075	0.083
pH	0.2688	0.2744
Alcohol	0.473	0.4077
Esters	1.381	1.261
Aldehyde	0.3993	0.4035
Dry matter content	0.4280	0.6248
Methanol	0.0064	0.006023
Reducing sugar	0.0968	0.1014
Protein solubility	2.346	1.658