EFFECT OF pH, *MURCHA* LEVEL AND FERMENTATION TIME ON SENSORY AND PHYSICOCHEMICAL PROPERTIES OF *BHATI JAND* PREPARED FROM BROKEN RICE

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Approval Letter

This ***dissertation*** entitled ***Effect of pH, Murcha Level and Fermentation Time on Sensory and Physicochemical Properties of Bhati Jand Prepared from Broken Rice presented*** by **Prakriti Gautam** has been accepted as the partial fulfillment of the requirements for the **B. Tech degree in Food Technology**

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(Prakriti Gautam)

Abstract

The objective of the present work was to study the effect of pH, *murcha* (amylolytic starter) level and fermentation time on sensory and physicochemical properties of *bhati jand* (rice wine) prepared from broken rice. *Jand* was prepared from broken rice using traditional starters with the variation in pH (3.0, 4.5, 6.0), *murcha* level (0.2,0.5 and 0.8%) and fermentation time (7,14 and 21 days). The sample formulation was done using DOE (Design Expert) v 13.0.1.0 which gave a total of 20 runs. *Jand* thus prepared was subjected to sensory (10 semi-trained panelists using a 5-point hedonic scale for appearance, smell, taste, sourness and overall acceptance), physicochemical (acidity, pH, TSS, reducing sugar, alcohol, esters, aldehyde, higher alcohol, methanol, proximate) and microbial analysis. Statistical analyses of the generated data were done using Design Expert v 13.0.1.1 for selecting the best combination, Genstat Discovery Edition 12 for two-way ANOVA at 5% significance level and MS Excel for graphical representation.

The statistical analysis of the sensory data showed that 0.2% *murcha* level, pH level of 6 and fermentation duration of 21 days resulted in *jand* with the highest sensory score whereas 0.8% *murcha* level, pH level of 6 and fermentation duration of 7 days resulted in *jand* with the lowest sensory score. Physicochemical analysis showed that methanol, ethanol, esters, aldehyde, higher alcohol, total soluble solids (TSS), acidity, pH and reducing sugar content for the *jand* with highest sensory score were 11 ppm, 8.89%, 831 g/100 L, 4.97 g/100 L, 68.5 ppm,14.25°Bx, 0.84%, 3.8 and 0.265% respectively *Jand* made at different pHs, levels of *murcha,* and fermentation durations differed significantly ($p<0.05$) with respect to sensory properties.

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Abbreviations	Full form
°Bx	Degree Brix
ANOVA	Analysis of Variance
AOAC	Association of Analytical Chemistry
FSSAI	Food Safety and Standards Authority of India
LAB	Lactic Acid Bacteria
LSD	Least Significant Difference
MYGP	Malt Yeast Extract Glucose Peptone
RH	Relative Humidity

Part I

Introduction

1.1 General introduction

Food fermentation is regarded as one of the oldest methods of food processing and preservation. More than anything else, man has known the use of microbes for the preparation of food products for thousands of years, and all over the world a wide range of fermented foods and beverages contributed significantly to the diets of many people (Achi, 2005). Fermented foods and beverages harbor diverse microorganisms from the environment, which include mycelial molds; yeasts; and bacteria, mostly lactic acid bacteria, bacilli, and micrococci. Microorganisms transform the chemical constituents of raw materials during fermentation and enhance the nutritive value of the products; enrich bland diets with improved flavor and texture; preserve perishable foods; fortify products with essential amino acids, health-promoting bioactive compounds, vitamins, and minerals; degrade undesirable compounds and anti-nutritive factors; impart antioxidant and antimicrobial properties; improve digestibility; and stimulate probiotic functions (Tamang and Kasipathy, 2010).

Most of ethnic fermented foods and beverages are produced by natural fermentation, except the alcoholic beverages in Asia, which are produced by using a consortium of microorganisms in the form of a dry, cereal-based starter. Diversity within the species or strains of several functional genera of dominant microorganisms has created ethnic foods with different sensory characteristics (Tamang and Kasipathy, 2010). *Jand* is a Nepalese indigenous fermented beverage prepared by solid-state fermentation of cereals using *murcha* (a traditional fermentation starter). It is one of the socially and culturally accepted mild alcoholic beverages and is presumably nutritionally superior to other alcoholic beverages, although its exact nutritional status is still unexplored. The term *murcha* is a Nepali word and the different ethnic communities of the region call it by their own dialect such as *khesung* by Limbu, *bharama* by Tamang, *bopkha* by Rai and *buth/thanbum* by Lepcha (Karki and Kharel, 2007).

Bhati jand is a Himalayan sweet–sour, mild alcoholic beverage consumed as a food, in the form of a paste, prepared from rice (Tamang, 2010). It is an inexpensive, high-calorie staple food consumed as a beverage by postnatal women and old people in villages who believe that it helps to regain their strength. The microbial analysis of *bhati jand* shows the yeast population to be at the level of 10^7 cfu/g whereas the LAB load is found in the range of 10^4–10^6 cfu/g). The yeast population is higher than that of LAB in *bhati jand*. Filamentous molds are absent in the final product (Thapa, 2001).*Bhati Jand* is prepared using native *murcha*, following the traditional method The alcohol content increases up to 10% during the fermentation of *bhati jand*. Maximum activities of saccharification and liquefaction of rice are observed on the third day of fermentation. *Saccharomycopsis fibuligera*, *Rhizopus* spp., and *Mucor* spp. contribute to the saccharification and liquefaction of glutinous rice, breaking the starch from substrates into glucose for alcohol production and also for aroma formation, during the preparation of *bhati jand* (Tamang and Thapa, 2006).

1.2 Statement of the problem

The basis of manufacturing of alcoholic beverages is alcoholic fermentation. To determine the quality of final product, control of fermentation is pre-requisite. In this context, fermentation monitoring is a growing need, which calls for fast, low-cost and non-destructive methods providing real-time or online information in order to assure an effective control on all stages of the process. Researches show that pH, inoculum size and fermentation time have great influence on final product quality of fermented beverages. Increase in inoculum size accelerates the fermentation rate but ethanol production was not affected. Concentration of higher alcohol was increased with increasing inoculum size (Erten *et al.*, 2006). Similarly optimum fermentation time leads to increase in productivity and quality of beverages. Yeast, molds and bacteria have their own optimum pH for fermentation. *Murcha*, on the other hand is a mixed starter culture of all yeast, mold and bacteria. It contains saccharifying molds, lactic acid bacteria and fermenting yeast. Although much researches have been done on industrial alcoholic beverages, the same on traditional alcoholic beverages are rare. In the case of *jand*, some researches have been made on finger millet *jand* (*kodo ko jand)* but researches regarding rice *jand* (*bhati jand*) is scanty. Being the stable crop of Nepal, it would be very helpful if optimum conditions could be established for the preparation of *jand* from rice. Furthermore, use of broken rice grains for *jand*

preparation serves in adding value to the broken grains which, otherwise would get very little price in the market.

1.3 Objectives

1.3.1 General objective

The general objective of dissertation work was to study the effect of pH, *murcha* level and fermentation time on physicochemical and sensory quality of *jand* prepared from broken rice.

1.3.2 Specific objectives

The specific objectives of the present study were:

1. To carry out analysis of the raw materials.
2. To prepare the *jand* varying the pH, *murcha* level and fermentation time.
3. To evaluate the sensory properties of *jand*.
4. To analyze the physicochemical properties of *jand*.

1.4 Significance of the study

Despite being economically feasible, traditional alcoholic beverages are not getting enough improvement and their production has been confined to household level only. So, researches must be carried out for their improvement in production methodology and raw materials composition. Although few researches have been carried on finger millet *jand* but researches on *bhati jand* is scanty. The findings of the present study can be used in improving the productivity and quality of traditionally prepared beverage. Furthermore, my research will be of great help in adding value to the broken rice grains which would, otherwise, get very little price in the market.

1.5 Limitations of work

1. Broken rice of mixed variety was used rather than pure variety (not available).
2. Marketed *murcha* sample was used as inoculum rather than isolated one.
3. Study was limited to small laboratory scale, which may not reflect the result one may get when carried out in commercial scale.

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 (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 AD, 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).

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), *bhati jand* (rice beer) (Tamang *et al.*, 1988b).

2.4 Some alcoholic beverages from cereals

Different cereals are used for the production of alcoholic beverage, the major types are mentioned in the following sub sections.

2.4.1 Alcoholic beverages prepared from millet

As cited by Pederson (1971), *tumba* 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. Similarly *braga* is a fermented drink prepared from millet in Romania (Pederson, 1971).

2.4.2 Maize

The early settlers in America were introduced with many types of beverages prepared with corn as the main ingredients. In 1587, Sir Walter Raleigh was introduced with *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 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, 1987). 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, 1987).

2.4.4 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 and 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 and

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 and may continue for 30-40 days. More rice and koji may be added to continue the fermentation. It is finally filtered, pasteurized and bottled (Pederson, 1971).

2.5 Broken rice

Rice is the main crop of Nepal as well as of the world. The main diet of the Nepalese is also rice. Fifty-five percent of the cultivated land of Nepal is covered with rice. Rice is cultivated in the diverse eco-climatic ranges of Nepal at differing altitudes, topography and climate. More than half of the human population depends on rice for food. Ninety percent of the rice grown in the world is produced and consumed in Asia. There are different views about the origin of rice by different scientists. As early as 1930 Villon pointed out that the origin of the present rice is in the South- East Himalayan region. Therefore, according to him the origin place of rice is in the South-East Asia, India, China and Indochina where different types of rice are found. If we consider his views the Himalayan range is also in Nepal. Rice belongs to gramineae family and *Oryza* genus. There are 25 species of rice, out of 25 species 23 are wild type and two species are cultivated. The rice of Asia is *Oryza sativa* and Europe, Africa and America are *Oryza glaberrima*. Rice cultivation has been done since the beginning of the civilization, so there are thousands of varieties in the world (Mallick., 1981).

Rice (genus *Oryza*) is a plant of the grass family, which is a dietary staple of more than half of the world's human population. Rice cultivation is well suited to countries with low labor costs and high rainfall, as it is very labor-intensive to cultivate and requires plenty of water for irrigation. However, it can be grown practically anywhere, even on steep hillsides. Rice is the world's third largest crop, behind maize (corn) and wheat. Although its species are native to South Asia and certain parts of Africa, centuries of trade and exportation has made it common place in many cultures. The modern English word 'rice' originates from ancient Greek word '*arizi*' which in turn was borrowed from the Tamil word of the same pronunciation, strongly indicating trade relationship between ancient Greeks and Tamils (Pokhrel, 2008). There are only two cultivated species *Oryza glaberimma* and *Oryza sativa*.

Oryza glaberimma is confined to West Africa but is being replaced by *Oryza sativa*. Morphologically, there are only small differences between these species. Origin of *Oryza sativa* is South-East Asia, particularly India and Indo-China (Aarathi *et al.*, 2003).

Rice cultivation is considered to have begun simultaneously in many countries over 6,500 years ago. Two rice varieties were domesticated: Asian rice (*Oryza sativa*) and African rice (*Oryza glaberrima*). It is believed that common wild rice, *Oryza rufipogon* was the wild ancestor of Asian rice. *O. sativa* appears to have originated around the foothills of the Himalayas, with *O. sativa* indica on the Indian side and *O. sativa japonica* on the Chinese side. African rice has been cultivated for 3,500 years (Pokhrel, 2008).

Rice has been one of the most commonly used grain products since ancient times. It is the staple food of the greatest number of people. Historian can't be accurate about the first appearance of rice because rice cultivation is older than recorded events. Though a lack of historical records prevents accurate determination, botanical evidence suggests strongly that rice originated in Southeast continental Asia. Rice is grown in all tropical countries in eastern and southern Asia including the larger nearby islands, especially Japan. The principal rice producing countries are China, India, Pakistan, Japan and Indonesia, Thailand, Indochina, Myanmar and Philippines also produce large quantities of rice. Many varieties of rice are produced throughout the world. Broken rice is separated after the polishing phase and has the same chemical composition as white rice. During milling an average of 50% brown rice then approximately 16% broken rice, 20% husk and 14% bran is produced. Further grains break before and after milling in transport. Mechanical separators are used to separate the broken grains from the whole grains. Broken rice may or may not have lower fiber and nutrient content, but generally has similar energy content to intact rice. The differences for a final user would be in the cooking results basically, not in the nutrient aspect. Broken rice can also be consumed as part of local cuisine in several countries in Africa, Thailand, and elsewhere in South East Asia, where the broken varieties are often less expensive so are preferred by lower income consumers (Johnson and Peterson, 1974).

2.5.1 Production of rice

In Nepal, 4.16 million metric ton of paddy is produced from 1.5 billion hectare cropping area. The productivity of paddy is increased from last 8 years. World production of 19 rice has risen steadily from about 200 million tons of paddy rice in 1960 to 600 million tons in

2000. Milled rice is about 68% of paddy rice by weight. In the year 2000, the top three producers were China (3% of the world production), India (21%), and Indonesia (9%). World trade figures are very different, as only about 5-6 % of rice produced is traded internationally. The largest three exporting countries are Thailand (26% of world exports), Vietnam (15%), and the United States (11%) while the largest three importers are Indonesia (14%), Bangladesh (4%) and Brazil (3%) (Pokhrel, 2008).

2.5.2 Nutritional value of broken rice (rice)

The composition of rice differs with the variety, the nature of the soil, environmental conditions and the fertilizers applied. The fat content of rice is low and most of it is removed in the process of milling and is contained in the bran (Grist, 1975). The nutrient content of rice is not affected when it is broken during processing. The average composition of both husked and polished broken rice is given in Table 2.1.

Table 2.1 Average composition of husked and broken rice

Proximate components	Husked	Polished (broken)
Carbohydrate (%)	87.67	90.79
Protein (%)	8.67	8.15
Fat (%)	2.45	0.37
Crude fiber (%)	0.88	0.16
Ash (%)	1.22	0.36

Source: Matthias, (1999)

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 (Tamang *et al.*, 1988b).

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). 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). Aidoo *et al.* (2005) have also reviewed some aspects of *jand* They have described the role of *mucaraceous* fungi in producing amylase needed to saccharify and liquefy starch.

The amylase activity has been reported to reach 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 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). Physicochemical properties of *jand* from different cereals is given in the Table 2.2.

Table 2.2 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 (°Bx)	1.75	0.48	2.61	0.38

Source: Upadhyaya (2005)

Several factors contribute to the overall sensory quality of *jand*. Some of them are shown in the Table 2.3.

Table 2.3 Factors affecting overall sensory quality of *jand*

Factors	Components
Raw materials	Cereal substrates such as finger- millet, wheat, rice, maize, etc. used for fermentation
Fermentation conditions	Temperature, pH, aerobicity, duration of fermentation, solid- state or submerged-state fermentation
Murcha quality	Species and strains of the essential microorganisms (yeasts and molds), presence of extracellular enzymes, amylase in particular
Physicochemical properties of *jand*	Alcohol content, acidity, pH, reducing sugars, esters and other congeners
Organoleptic properties of *jand*	Taste, smell, mouth- feel and color

Source: Rai (2006a)

2.6.2 *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; 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 feints and foreshots for improving quality of *raksi*.

2.6.3 *Hyaun thon*

Hyaun thon is an alcoholic beverage (undistilled) indigenous to Nepal. The preparation methodology of *hyaun thon* is entirely different from another 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 (Rai, 2006b).

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. 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).

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).

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

1. Product yield may be higher.

2. 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.

3. Mixed cultures are able to bring about multistep transformations that would be impossible for a single microorganism.

4. 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.

5. 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.

6. Unskilled people with a minimum of training can maintain mixed cultures indefinitely. 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.

7. 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.

8. 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

1. Scientific study of mixed cultures is difficult. Obviously, it is more difficult to study the fermentation if more than one microorganism is involved.

2. Defining the product and the microorganisms employed becomes more involved in patent and regulatory procedures.

3. Contamination of the fermentation is more difficult to detect and control.

4. 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.

5. 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

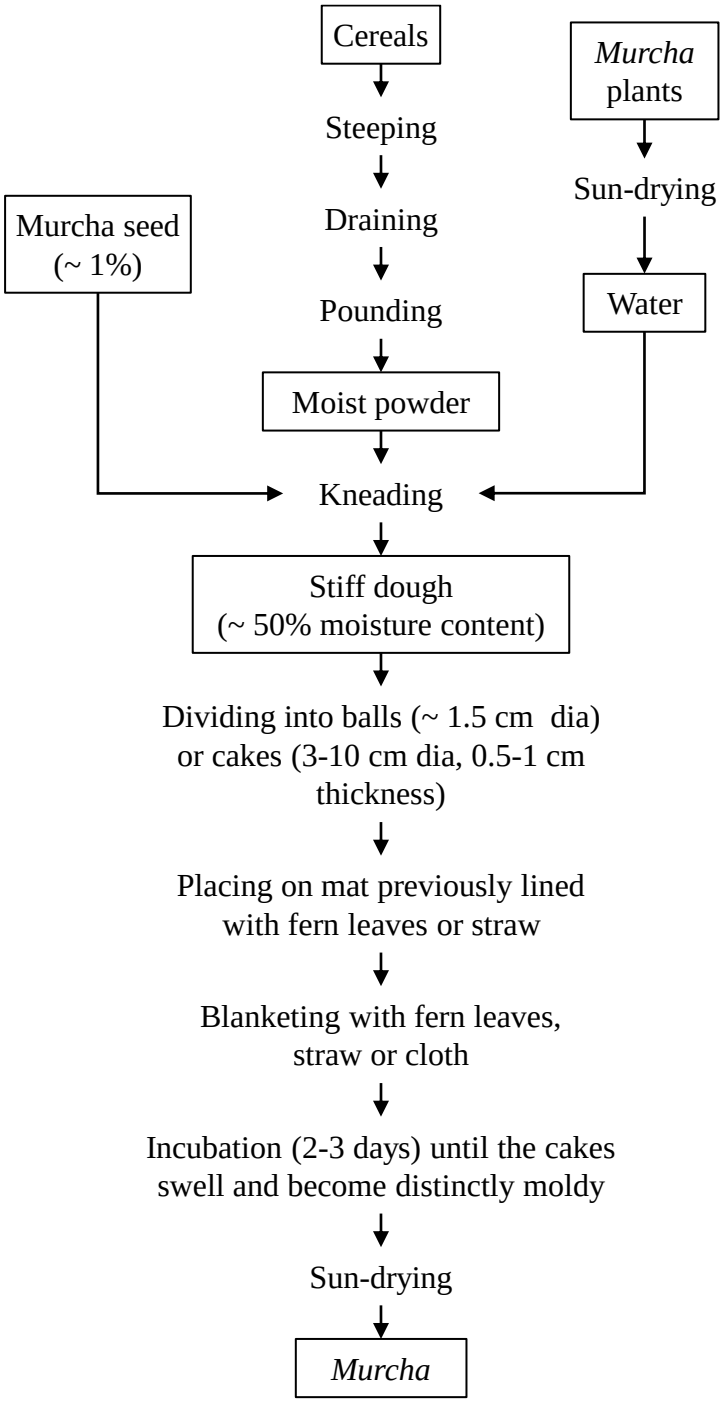
2.9.1 *Murcha*

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*. *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 (Gajurel and Baidya, 1979).

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. Fig. 2.1. shows the general method of preparation of traditional *murcha*.



Source: Subba *et al.* (2005)

Fig. 2.1 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.*, 1988b). The yeast population in these samples was very high ranging from 5.4. $\times 10^6$ to 6.1 $\times 10^8$ cfu/g and mold count were 1.5 $\times 10^6$ to 2.8 $\times 10^8$ cfu/g. The identified yeasts were *Saccharomycopsis fibuligera*, *Pichia anomala,* and *Saccharomyces* sp.

The *murcha* samples were also collected more recently in year 2000 from Nepal and the microbiological study carried out (Shrestha and Rati, 2002) Yeast and lactics were present in high numbers in *manapu* starters, whereas molds were dominant in wheat-based m*ana* samples. In general, *manapu* 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. around forty-five strains of yeasts, 29 strains of lactic acid bacteria, and 21 cultures of fungi were isolated and purified from various *murcha* samples. Among the yeast, *Saccharomyces cerevisiae* strains were found to be dominant followed by *Candida versatilis* (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) (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 25ºC.

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* to *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 the 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 (Tamang *et al.*, 1988a). Molds were dominant in wheat-based mana samples. The m*ana* starters contained molds as dominant flora recording more than 1×10^7 cfu/g (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) (Platt, 1994).

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 and 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 is present causing many simultaneous reactions 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. The final concentration of ethanol depends on the initial concentration of sugars (or other final concentration of ethanol depends on the initial concentration of sugars (or other substrate) 9 in the must or juice, as well as the fermentation temperature, since some ethanol is lost during warmer, faster fermentations (Buglass, 2011) A truncated form of the metabolic pathway for ethanol synthesis is given in Fig. 2.2.

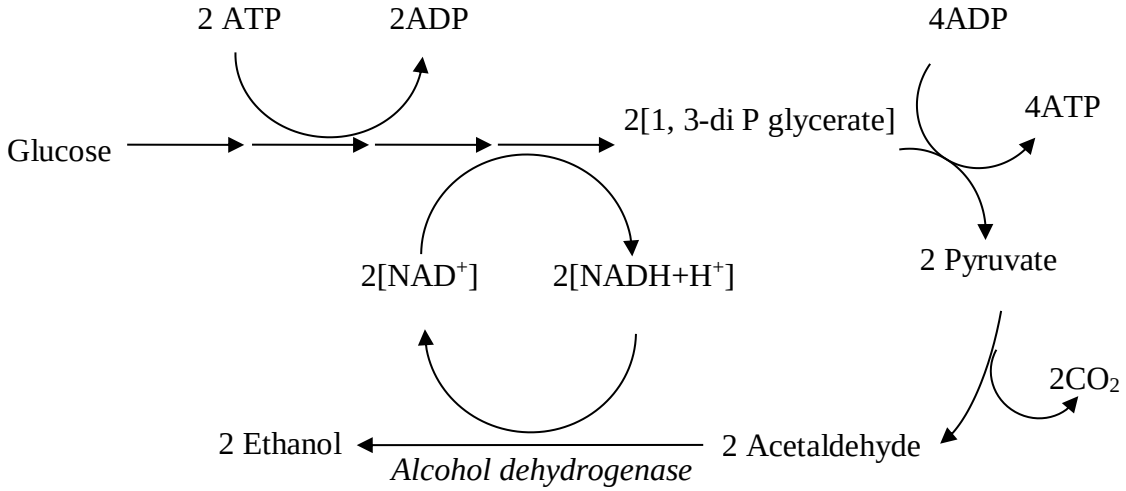


Fig. 2.2 Simplified pathway of alcohol synthesis by yeast

2.12 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).

2.12.1 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 principal 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, 2006a). 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) to produce pediocin (an antibiotic) that is inhibitory to pathogens, *Listeria* in particular organisms.

2.12.2 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* and *kinema* (Karki *et al.*, 2005).

2.13 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. 2.3.

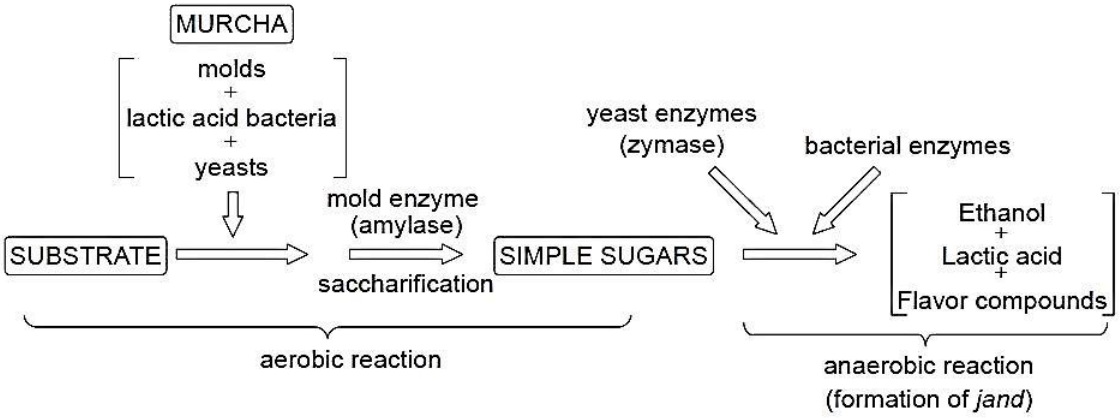


Fig. 2.3 Sequential and concerted action of *murcha* flora on cereal substrate

2.13.1 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. The chemical reaction of conversion is shown in the Fig. 2.4.

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

Fig. 2.4 Conversion of glucose to ethanol and carbon dioxide

2.14 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).

2.14.1 Esters

Esters are numerically the largest group of organoleptic compounds in alcoholic beverages. Lower esters have pleasant odors that are usually described as fruity. Although some ester formation may occur during the distillation of spirits, the most common esters are produced

by the yeast during fermentation stage (Barnett *et al.*, 1990). 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 content at up to 50 mg/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 *et al.*, 1990).

2.14.2 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).

2.14.3 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 tricarboxylate acid cycle. Pyruvate itself constitutes a qualitatively important group of acids. They may have direct effect on flavor (e.g., the mouthfeel 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 lost 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).

2.14.3.1 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 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. 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 of pathway (also called as 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).

2.15 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 cerevisiae*, 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).

The basic principle of toxicity of methanol is that it is metabolized primarily in liver and kidney by oxidation of formaldehyde and formic acid The metabolic pathway is shown in the Fig. 2.5.

Fig. 2.5 Conversion of methanol to formaldehyde and formic acid

2.16 Toxic effect

Major toxic effects are caused by formaldehyde and formic acid. The formic acid is responsible for the damage of retinol cells that may cause blindness while the latter produces severe acidosis that may be eventually led to death. A minor effect of methanol is depression of central nervous system. 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 50 mg/L may be discounted. The fatal internal dose of methanol is 60-250 ml. The exposure limit is 200 mg/L (Boing, 1987).

2.17 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 and Solid-state fermentation. 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).

2.17.1 Role of water in solid state fermentation

1. 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.
2. 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).
3. In the same way, if the transfer of water induced by water stress leads to a denaturation of the mechanical structure of the plasmid membrane, all the properties of permeability and transport through the membrane are affected and the cell is then perturbed (Loecker *et al.*, 1978).

2.17.2 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 and usage of starchy agricultural wastes makes the whole process more economical (Couto *et al.*, 2001).

2.18 Effect of variation in pH, fermentation time and inoculum rate on physico chemical properties of fermented beverage

A study on the mulberry fruit wine shows that the optimal condition for mulberry fermentation was defined as pH 3.2, inoculum size 0.53%, fermentation temperature of 31.4℃ and fermentation time of 6 days (Wang *et al.*, 2017).

Similar study in white wine shows that the fermentation rate was improved with higher amounts of yeasts, but ethanol production was not affected. Concentrations of higher alcohols increased with the increasing inoculum levels. The amount of ethyl acetate was reduced with increased inoculum levels (Berry and Chamberian, 1986). The evaluation for TSS and pH of guava fruit wine shows that both pH and TSS follows a decreasing trend with the increase in fermentation period (Singh and Samsher, 2020).

The biochemical composition of wine prepared from *ambia bahar* fruits of Nagpur mandarian indicated that the levels of wine yeast inoculum used as 3,6 and 9% and levels of pH 3.0, 3.5, 4.0, 4.5 and 5.0 affected the wine quality. The wine prepared with 6 per cent yeast inoculum and pH 4.0 of must yielded higher alcohol content followed by wine prepared with 6% yeast and 3.5 pH of must (Kadu *et al.*, 2021).

Part III

Materials and methods

3.1 Materials

Broken rice of mixed variety and *murcha* cake were collected from local market of Dharan. Media needed for all microbiological works were obtained from Central Campus of Technology, Dharan. All the necessary chemicals, utensils, equipment and glassware needed for the work were obtained from the campus.

3.2 Methods

3.2.1 Sample preparation

After collection, the samples were packed in reclosable low density polyethylene plastic pouches, taking care to avoid cross contamination and stored in bulk in the refrigerator until needed. *Murcha* cakes were mixed uniformly and placed in clean polyethylene plastic puoches avoiding the contamination.

3.2.2 Formulations of *jand* samples

Design Expert 13.0.1.0, a statistical software, was used for creating the design space (experimental plan). Table 3.1 shows the treatments and total formulated samples.

Table 3.1 Experimental plan (design space)

Treatments	A*: Murcha*	B: Cooking water pH	C: Fermentation time
	%	units	Days
H	0.5	4.5	7
B	0.8	3	21
C	0.8	6	7
E	0.2	6	7
I	0.2	4.5	14
F	0.5	3	14
L	0.8	4.5	14
M	0.5	4.5	21
J	0.2	3	21
G	0.5	4.5	14
O	0.8	6	21
A	0.2	6	21
D	0.2	3	7
K	0.5	6	14
N	0.8	3	7

3.2.3 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 g *murcha* powder per 100 g cooked rice) for 1 week and tentative organoleptic appraisal of the product was done by panelist.

3.2.4 Adjusting the cooking water pH

Water of desired pH (3, 4.5 and 6) was prepared by using 2% citric acid solution.

3.3 General method of preparation of *jand*

3.3.1 Preparation of raw materials

Broken rice was cleaned and washed with water. Then it was divided into three different parts. Water of three different pHs (3, 4.5 and 6) was added in rice in three different vessels and then cooked under the same cooking conditions till soft consistency (about 20 min) at 100°C and cooled to room temperature.

3.3.2 Cooking of rice

The purpose of cooking rice grains is to convert the starch into amylose and to denature the protein in the rice. Steaming also destroys the contaminating micro-organisms. Cooking of rice grains was performed in an open vessel. The cooked rice was spread onto a muslin cloth and allowed to air cooled.

3.3.3 Inoculation and fermentation

Cooked rice was divided into 20 equal parts in clean plastic vessels of equal size. Respective amount of *murcha* (0.2%, 0.5% and 0.8%) was added as per the recipe formulation in design expert in the form of powder uniformly over the surface of the cooked and cooled mashes. 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 48 h at aerobic condition with loose covers. After 48 h, optimum biomass was seen in visible puffy colonies. Then the vessels' covers were tightly closed and left undisturbed at room temperature for 7, 14 and 21 days for alcohol fermentation.

3.3.4 Preparation of *bhati jand*

For the preparation of *bhati jand*, 1.5 parts (by vol.) of previously boiled and cooled water was added to each part (by wt.) of biomass developed rice used for alcoholic fermentation. The overall experimental detail is given in Fig. 3.1.

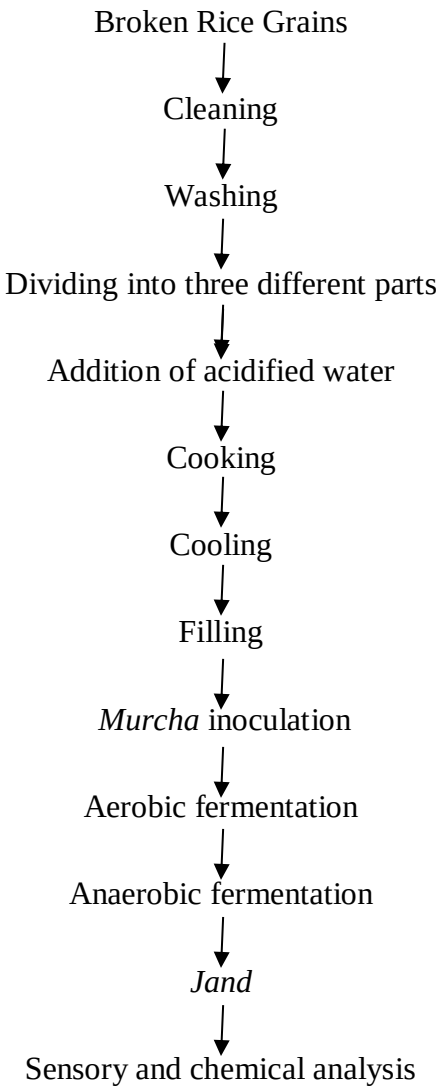


Fig. 3.1 Preparation of *bhati jand*

3.4 Sensory evaluation and physicochemical analysis of the product

3.4.1 Sensory evaluation

Sensory evaluation of *bhati jand* was done using 10 semi-trained panelists consisting of B.Tech (Food) students and teachers on a five-point hedonic rating (5 = like extremely, 1 = dislike extremely) for appearance, taste, sourness, smell, and overall quality) of the *jand*.

They were asked to rate the product according to their liking or disliking following 5 points hedonic rating test as described (Ranganna, 1986).

3.4.2 Physicochemical analysis

Fermented mash (*jand*) was taken to determine its pH and TSS. 10 g 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. 200 g of fermented mash was taken for neutralization. After neutralization, it was then taken for distillation and the distillate was used for alcohol determination. After that, same distillate was divided equally (about 100 ml) for the determination of ester, aldehyde, methanol, higher alcohol and reducing sugar (FSSAI, 2019).

3.4.2.1 pH

pH of the samples was 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°C.

3.4.2.2 Total Soluble Solids (TSS)

TSS of different samples were measured by using the refractometer (Hanna make, Portugal). The refractometer was thoroughly washed with distilled water and then wiped with soft tissue after each test.

3.4.2.3 Reducing sugar

Reducing sugar was determined by Lane and Eynon method on spent wash recovered after distillation of alcohol and the values were expressed as % of dextrose (FSSAI, 2019).

3.4.2.4 Alcohol content

Alcohol content was determined by pycnometric method (AOAC, 2005) taking 200 g of *jand* (mash) and the values were expressed in % (v/v).

3.4.2.5 Acidity

Acidity was determined on 10 g mash by titrimetric method (Ranganna, 1986) using 0.1*N* sodium hydroxide and the values were expressed in % (m/v) as lactic acid.

3.4.2.6 Methanol content

Methanol contents of the samples were determined by colorimetric method described in (AOAC, 2005). Briefly, 2 ml of $KMnO_4$ solution (3 g $KMnO_4$ dissolved in a mixture of 15 ml H_3PO_3 and 85 ml distilled water) was pipetted into a 50 ml volumetric flask, chilled in ice bath. 1 ml of the distillate sample was added to the flask and stand for 30 min in ice bath. The excess of $KMnO_4$ solution was decolorized with 2% sodium sulphite solution and 1 ml chromotropic acid solution (5% aqueous solution) was added. Then 15 ml conc H_2SO_4 was added slowly with swirling and placed in hot water bath maintained at 70°C for 15 min and cooled. The volume was made up to 50 ml, and the absorbance was read at 575nm against a reagent blank containing 5.5% ethanol treated similarly. Standard methanol solution (0.025% by volume in 5.5% ethanol) was also treated simultaneously in the same manner, and the absorbance recorded. Methanol content in the *jand* was calculated as follows:

Methanol content (% v/v) = Sample absorbance × 0.025 / Standard absorbance

3.4.2.7 Aldehyde content

Aldehyde content was determined as per the method described (FSSAI, 2019) and the values were expressed in g per 100L of absolute alcohol as acetaldehyde.

Briefly, 50 ml of distilled liquor was taken in a 250 ml Iodine flask and 10 ml of sodium bisulphite solution was added to it. The flask was then put in a dark place for 30 min with constant shaking. 25 ml of standard iodine solution was added to it and back titrated the excess iodine against standard thiosulphate solution using starch indicator to light green end point. Blank was run similarly taking 50 ml distilled water. The difference in titer value in milliliters of sodium thiosulphate solution gave the equivalent aldehyde content.

Aldehyde expressed as acetaldehyde, g per 100 L of absolute alcohol is

$$\frac{V\times 0.0011\times 100\times 1000\times 2}{V1}$$

Where, V1= alcohol % by volume

V= difference in titer of blank and sample, in ml of sodium thiosulphate solution

3.4.2.8 Esters content

Ester value of distillate was determined as per FSSAI manual (FSSAI, 2019) and the values were expressed in g per 100 L.

Briefly, 10 ml standard NaOH was added to the neutralized distillate and refluxed in the steam bath for an hour. Then it was cooled and unspent alkali was back titrated against standard sulphuric acid. Blank titration was simultaneously carried out taking 50 ml of distilled water instead of the distillate in the same way. The difference in the titer value in milliliters of standard sulphuric acid gave the equivalent ester.

Esters expressed as ethyl acetate, g per 100 L of absolute alcohol is

$$\frac{V\times 0.0088\times 100\times 1000\times 2}{V_1}$$

Where, V= difference of the titer value of standard H_2SO_4 used for blank and sample, in ml

V1= alcohol % by volume

3.4.2.9 Higher alcohol content

Higher alcohol content was determined by spectrophotometric method described in (AOAC, 2005). Briefly, 1 g of the fusel oil standard (4 volumes isoamyl alcohol mixed with 1 volume of isobutyl alcohol) was diluted to 1 L with water. Finally, working standard solutions were prepared by pipetting 0, 5, 10, 25 and 35 ml of fusel oil standard solution in to 100 ml volumetric flask containing 7 ml of 95% neutral ethanol and dilution to volume with distilled water. Distillate (1 ml) was pipetted in a test tube and diluted to 2 ml with distilled water.

One milliliter of DMAB (9 p- Dimethylaminobenzaldehyde) solution (1 g DMAB dissolved in a mixture of 5 ml H_2SO_4 and 90 ml distilled water and volume made up to 100 ml with distilled water) was added to the test tube, shaken and placed in ice bath for 3 min. With the tube still in ice bath, 10 ml of chilled H_2SO_4 was added into the tube, shaken and replaced in ice bath for 3 min. Then the tube was placed in a boiling water bath for 20 min and replaced in ice bath for 5 min. The tube was shaken and brought to room temperature. Similar procedure was followed for fusel oil working standard solutions. Transmittance (% T) of both the test sample and working standard solutions were read at 540 nm against reagent blank as reference. The concentration of the fusel oil was found out from the fusel oil standard curve prepared by plotting gram fusel oil on linear as abscissa against % T as ordinate on log scale of semi log paper. The results were expressed as mg fusel oil/ 100 ml *jand.*

PART IV

Results and discussion

Broken rice was brought from the local market of Dharan. Proximate analysis of broken rice was performed. After cleaning, washing and cooking the broken rice, *Bhati jand* was prepared varying the cooking water pH level, amount of *murcha* and duration of fermentation. PET vessels of density 1.38 g/cm^3 were used for the fermentation. All vessels were placed under identical condition throughout the process. After completing the fermentation, sensory analysis was done for observation of the effect variation.

4.1 Proximate composition of broken rice.

The proximate analysis of broken rice was performed in laboratory are tabulated in Table 4.1

Table 4.1 Proximate composition of broken rice

Proximate composition (wet basis)	Percentage
Moisture	15.6±0.4
Crude protein	7.2±0.15
Crude fiber	0.69±0.01
Ash content	0.53±0.02
Fat	0.33±0.02
Carbohydrate	75.56±0.23

* Values are the means of three determinations.

The mean value (%) of moisture content, ash, protein, fat, crude fiber and carbohydrate for broken rice flour was found to be 15.6%, 0.53%, 7.20%, 0.33%, 0.69% and 75.56% respectively. HMG-N (1986) reported that the moisture content, crude protein, total ash, fat, total carbohydrate and crude fiber to be 13.7%, 6.8%, 0.6%, 0.5%,78.2% and 0.2%

respectively. Similarly, according to Acharya, (1999), the moisture content, crude protein, total ash, fat, total carbohydrate and crude fiber of rice is 10.9%, 7.1%, 1.2%, 2.4%, 77.1% and 0.9% respectively. Similar kind of observation can be seen in this work.

4.2 Microbial profile of *murcha*

The microbiological parameter of *murcha* were determined which is tabaulated at Table 4.2. the *murcha* samples to be used were mixed thoroughly to obtain the uniform microbial count. The same was then taken for the microbial analysis. The yeast content in *murcha* was 23x 10^{-7} cfu/g and the molds content was counted to be 1×10^{-7} cfu/g.

Table. 4.2. Microbial analysis table of *murcha* sample

Sample	Yeast (cfu/g)	Mold (cfu/g)
Murcha	23x 10^{7}	1×10^{7}

According to (Rai, 2016), molds count has been found to be more than the molds count in murcha and the similar observation can be seen in this work.

4.3 Effects of variation of pH, *murcha* level and fermentation time on sensory attributes of *jand*

Fifteen different samples were taken for sensory analysis by 10 semi- trained panelists using a 5-point hedonic rating scale. The bases of sensory analysis were appearance, sourness, smell, taste and overall acceptance. The obtained data from sensory analysis were analyzed using two-way ANOVA at 5% level of significance to study the significance difference among the formulation made.

4.3.1 Appearance

The mean sensory score ±standard deviation for appearance of 15 samples A, B, C, D, E, F, G, H, I, J, K, L, M, N, and O were found to be 4.7, 4.4, 3.9, 3.8, 4, 4.4, 4.6, 4.1, 4.6, 4.8, 4.6, 4.7, 4.4, 4.3, 4.1 respectively. The statistical analysis showed that pH variation, amount of *murcha* used and duration of fermentation had significant effect on appearance of *jand* at 5% level of significance. Mean scores showing the effect of variation of pH, *murcha* level and fermentation duration on appearance in Fig. 4.1.

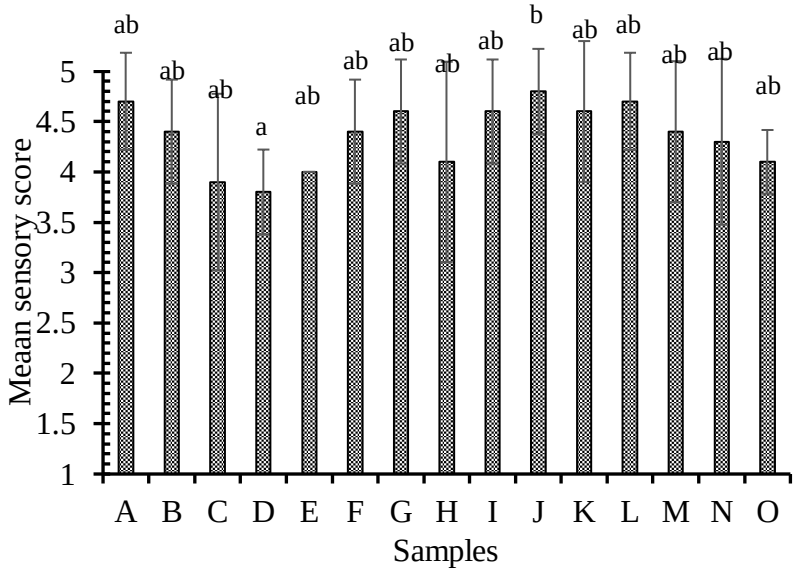


Fig. 4.1 Effects of variation of pH, *murcha* level and fermentation duration on appearance

Sample J was found to be having the maximum sensory score which was made with 3 pH, 0.2 % *murcha* level, 21 days of fermentation whereas the lowest sensory score was given to the sample D which was made with pH 3, 0.2 % *murcha* level and 7 days duration of fermentation.

Since there are no coloring pigments in rice, the role of pH on appearance is seen negligible. However, on comparing the highest and lowest ratings on appearance, it can be seen that samples having equal amount of *murcha* level and equal cooking pH shows the highest and lowest rating due to the difference in duration of fermentation. Sample J, having highest days of fermentation was found to be having the maximum appearance rating as compared to sample D, which has the lowest rating and has the shortest duration of

fermentation. This shows that duration of fermentation has a great role in appearance, the greater duration giving the better appearance.

4.3.2 Sourness

The mean sensory score ±standard deviation for sourness of 15 samples A, B, C, D, E, F, G, H, I, J, K, L, M, N, O were found to be 4.9, 3.5, 2.5, 2.8, 4.2, 4.3, 4.4, 3.4, 3.6, 4.3, 3.6, 4, 4.3, 3.7, 3.1 respectively. The statistical analysis showed that pH variation, amount of *murcha* used and duration of fermentation had significant effect on sourness of *jand* at 5% level of significance. Mean scores showing the effect of variation of pH, *murcha* level and fermentation time on sourness is given in Fig. 4.2.

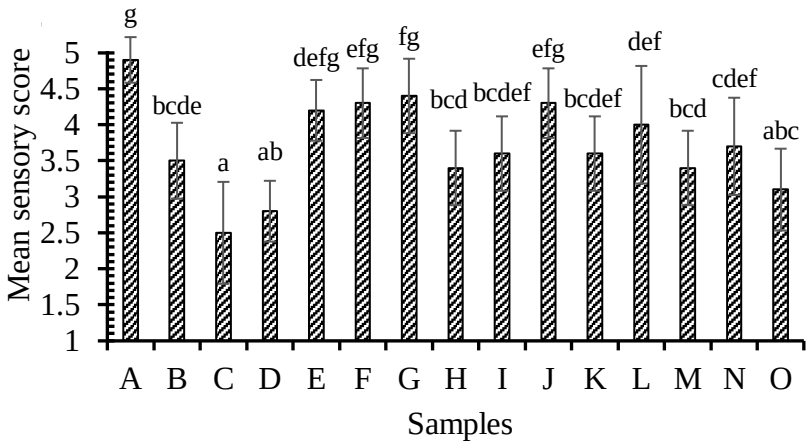


Fig. 4.2 Effects of variation of pH, *murcha* level and fermentation duration on sourness

The sample A got the highest sensory score which was made with 6 pH, 0.2% *murcha* level, and 21 days of fermentation whereas the lowest sensory score was got by C which was made with 6 pH, 0.8 *murcha* % and 7 days duration of fermentation.

Must fermented at the lower pH gives the highest percentage of alcohol and titrable acidity (Satav and Pethe, 2016).

Comparison of sample A and sample C, with maximum and minimum scores in sourness shows that despite having same initial cooking pH, showed the significant difference in sourness rating. This is due to variation in *murcha* level and fermentation days, A having the lowest inoculum and highest duration of fermentation and C having just opposite to A. Hence

this research shows that inoculum size and duration of fermentation also have significant effect in acidity and thus sourness.

4.3.3 Smell

The mean sensory score ±standard deviation for smell of 15 samples A, B, C, D, E, F, G, H, I, J, K, L, M, N and O were found to be 4.9, 4.4, 3.4, 3.7, 4, 3.2, 4.6, 3.7, 3.7, 4.1, 3.8, 4.3, 4.1, 4.2, 3.3. The statistical analysis showed that pH variation, amount of *murcha* used and duration of fermentation had significant effect on smell of *jand* at 5% level of significance. Mean scores showing the effect of variation in pH, fermentation duration and *murcha* level on smell is given in Fig. 4.3.

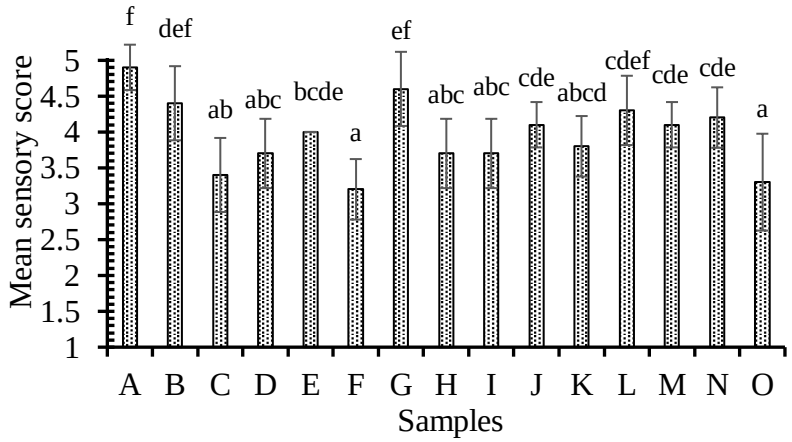


Fig. 4.3 Effects of variation of pH, *murcha* level and fermentation duration on smell

The inoculum size has the significant effects on the aroma compound in wine making , consistent increase of desired aroma compounds (esters, lactones, and free monoterpenes), and decrease in less desired compound for white wine (higher alcohols and medium chain fatty acids) was seen with increase in number of inoculum size (Carrau *et al.*, 2010).

Similar kind of result is seen in this research work. Increase in inoculum size led to increase in flavor components but the duration of fermentation also played significant role in the production of flavor compounds. Sample A, despite having low inoculum size had the highest rating in smell than sample C, with high inoculum size as A had the longest duration of fermentation than C which had the shortest duration of fermentation.

The sample A got the highest sensory score which with the formulations of 6 pH, 0.2% *murcha* level, and 21 days of fermentations whereas the lowest sensory score was got by sample F with the formulations of 3 pH, 0.5% *murcha* level and14 days duration of fermentation.

4.3.4 Taste

The mean sensory score ±standard deviation for taste of 15 samples A, B, C, D, E, F, G, H, I, J, K, L, M, N, O were found to be 4.9, 4.2, 2.5, 3.6, 4.2, 3.8, 4.5, 3.7, 3, 4.4, 3.3, 4.3, 3.2, 3.3, 3.2 respectively. The statistical analysis showed that pH variation, amount of *murcha* used and duration of fermentation had significant effect on taste of *jand* at 5% level of significance. The mean scores showing the effect of variation of pH, *murcha* level and fermentation duration on taste is given in Fig. 4.4.

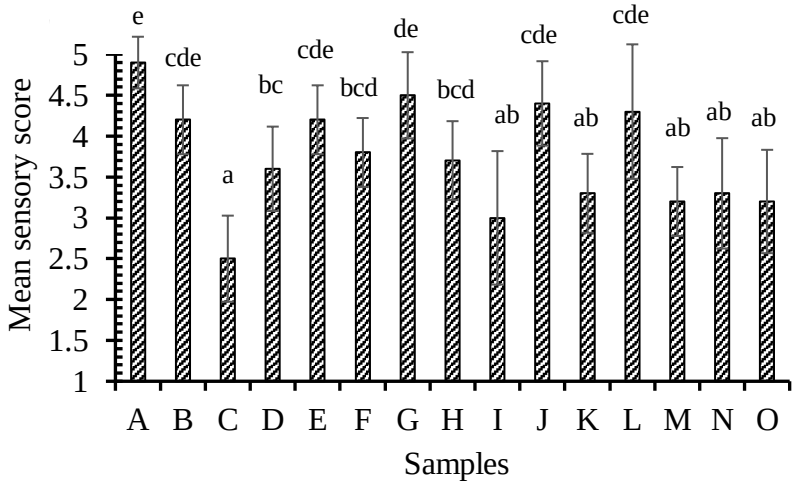


Fig. 4.4 Effects of variation of pH, *murcha* level and fermentation duration on taste

The sample A got the highest sensory score with the formulation of 6 pH, 0.2% amount of *murcha* level, 21 and days of fermentations duration whereas the lowest sensory score was got by the sample C which contained 6 pH, 0.8 % *murcha* level and 7 days duration of fermentation.

Alcohol and acidity are the major factor affecting the taste of *jand*. The optimum alcohol content and acidity was obtained with the pH level slightly higher than 3 and inoculum with more than 0.2% in wine sourness.

This study found out that sample A and sample C having the maximum and minimum rating in taste scores respectively have same pH but the duration of fermentation and *murcha* level are far different. On keeping the minimum level of *murcha* and letting to ferment for maximum days led to the maximum score in taste unlike C which had maximum inoculum level with minimum days of fermentation which might be due to low level of reducing sugar and high production of alcohol and acid on A compared to C.

4.3.5 Overall

The mean sensory score ±standard deviation for overall acceptance of 15 samples A, B, C, D, E, F, G, H, I, J, K, L, M, N, O were found to be 4.9, 3.9, 2.9, 3.4, 3.5, 3.8, 4.3, 3.7, 3.6, 4.3, 3.8, 4.7, 3.9, 3.7, 3.3 respectively. The statistical analysis showed that pH variation, amount of *murcha* used and duration of fermentation had significant effect on overall acceptance of *jand* at 5% level of significance. The mean scores showing the effect of variation of pH, *murcha* level and fermentation duration on overall acceptance is given in Fig. 4.5.

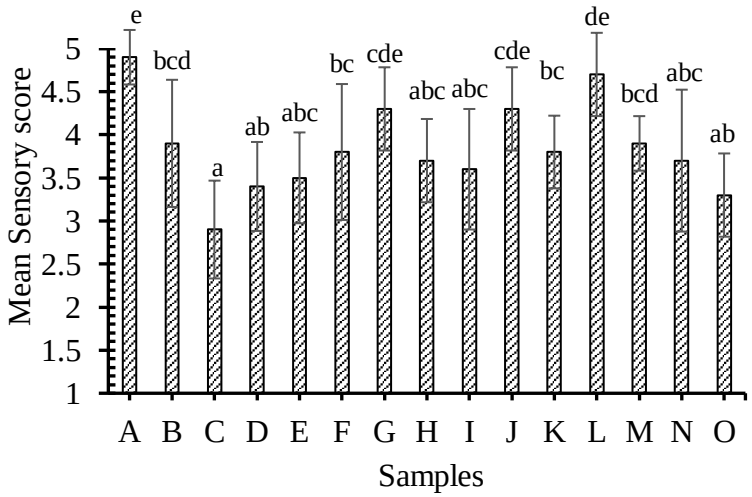


Fig. 4.5 Effects of variation of pH, *murcha* level and fermentation duration on overall acceptance

The sample A got the highest sensory score which had the formulations of 6 pH, 0.2% amount of *murcha* level, and 21 days of fermentations whereas the lowest sensory score was got by sample C which had the formulations of 6 pH, 0.8 % *murcha* and 7 days duration of fermentation respectively.

As compared to other samples, sample A had better sensory attributes such as appearance, smell, sourness and it got the highest sensory score in overall but the sample C got the lowest score due to the poor appearance, smell, sourness and taste.

4.4 Comparison of the samples with maximum and minimum sensory score in chemical composition.

Chemical analysis of the samples was analyzed. Table 4.3 shows the result of analysis.

Table 4.3 Chemical composition of samples with maximum sensory score (Sample A) and with minimum sensory score (Sample C)

Chemical composition	Sample A	Sample C
Methanol (ppm)	$11^{a}\pm0.5$	$47.11^{b}\pm0.15$
Ethanol (%)	$8.89^{a}\pm0.1$	$6.69^{b}\pm0.25$
Ester (g/100 L)	$831^{a}\pm0.09$	$533.5^{b}\pm0.36$
Aldehyde (g/100 L)	$4.97^{a}\pm0.025$	$3.15^{b}\pm0.02$
Higher alcohol (ppm)	$68.5^{a}\pm0.2$	$69.06^{a}\pm0.02$
TSS (°Bx)	$14.25^{a}\pm0.01$	$16.10^{b}\pm0.02$
Acidity (as lactic acid) %	$0.84^{a}\pm0.01$	$0.615^{b}\pm0.01$
pH	$3.8^{a}\pm0.01$	$3.75^{a}\pm0.01$
Reducing sugar (as dextrose) %	$0.265^{a}\pm0.01$	$0.58^{a}\pm0.01$

* Values are the means of three determinations

Methanol content of sample A was found to be 11 ppm whereas of sample C was found to be 47.11 ppm. Methanol content sample A and sample C was significantly difference ($P<0.05$). Similarly, ethanol content, ester and aldehyde were also significantly different ($P<0.05$) in sample A and sample C. The ethanol content, ester, aldehyde, TSS, acidity, reducing sugar of sample A were 8.89%, 831 g/100 L, 4.97 g/100 L,14.25,0.84, 0.265. However, the ethanol, ester, aldehyde, TSS, acidity, reducing sugar in sample C were 6.69%, 533.5 g/100 L, 3.15 g/100 L, 16.10, 0.615, 0.58. However, higher alcohol and pH content found to be almost same in both the samples, which were not significantly different. ($p<0.05$). The values of higher alcohol and pH in sample A and sample C were 68.5 ppm and 69.06 ppm, and 3.8 and 3.75 respectively.

4.5 Comparison of the samples with maximum and minimum scores in sensory attributes

The mean sensory scores showing the comparison of the two samples in terms of sensory attributes is given in Fig. 4.6.

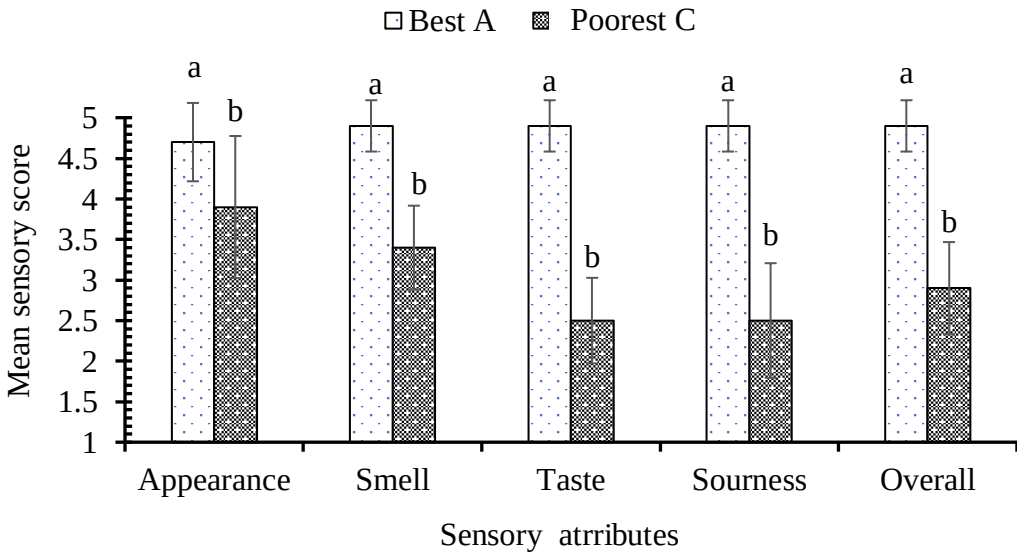


Fig. 4.6 The comparison of samples with highest score (sample A) and the sample with lowest score (sample C) in terms of sensory attributes

The mean sensory score of sample A for appearance was 4.7 and for smell, taste, sourness and overall was 4.9. The sensory attributes were significantly different ($p>0.05$) in these two

samples. The mean sensory score of sample C were 3.9,3.4,2.5,2.5 and 2.9 for appearance, smell, taste, sourness and overall, respectively.

Part V

Conclusions and recommendations

5.1 Conclusions

On the basis of work done, following conclusions can be drawn:

1. *Jand* prepared by maintaining 6 pH, 0.2% *murcha* level and 21 days fermentation duration had the highest sensory score while the *jand* prepared by maintaining 6 pH, 0.8% *murcha* level and 7 days fermentation duration had the lowest sensory score.
2. *Jand* made from different pH, level of *murcha* and fermentation duration differ significantly (at 5% level of significance) with respect to sensory properties.
3. Analysis of *jand* with maximum sensory score showed that it had 11 ppm methanol, 8.89% ethanol, 831 g/100 L ester, 4.97 g/100 L aldehyde, 68.5 ppm higher alcohol, 14.25°Bx TSS, 0.84% acidity, 3.8 pH and 0.265% reducing sugar.
4. However, analysis of *jand* with minimum sensory score showed that the worst sample had 47.11 ppm methanol, 6.69% ethanol, 533.5 g/100 L ester, 3.15 g/100 L aldehyde, 69.06 ppm higher alcohol,16.10°Bx TSS, 0.615% acidity, 3.75 pH and 0.58% reducing sugar.

5.2 Recommendations

The experiment can be further continued with the following recommendations:

1. The shelf life of *Jand* can be analyzed.
2. Entrepreneur can produce the *jand* with optimum sensory attributes with the formulation of 6 pH, 0.2% level of *murcha* and 21 days duration of fermentation.

Summary

With the motive of finding the optimum conditions for *jand* making, attempt was made to study the effect of pH, *murcha* level and fermentation duration on physicochemical and sensory quality of *bhati jand preparation of bhati jand* was carried out from broken rice using traditional starters with the variation in cooking water pH (3.0, 4.5, 6.0), *murcha* level (0.2,0.5 and 0.8%) and fermentation time (7,14 and 21 days). The sample formulation was done using DOE (Design- Expert) v 13.0.1.0 which gave a total of twenty runs. *Jand* thus prepared was subjected to sensory (10 semi-trained panelists using a 5-point hedonic scale for appearance, smell, taste, sourness and overall acceptance) and physicochemical (acidity, pH, TSS, reducing sugar, alcohol, esters, aldehyde, higher alcohol, methanol, proximate) and microbial analysis. Statistical analyses of the generated data were done using Design Expert v 13.0.1.1 for selecting the best combination, Genstat v 12 for ANOVA and post hoc and MS Excel for graphical representation.

The statistical analysis of the sensory data showed that 0.2% *murcha* level, pH level of 6 and fermentation duration of 21 days resulted in *jand* with the highest sensory score whereas 0.8% *murcha* level, pH level of 6 and fermentation duration of 7 days resulted in *jand* with the lowest sensory score. Physicochemical analysis showed that methanol, ethanol, esters, aldehyde, higher alcohol, total soluble solids (TSS), acidity, pH and reducing sugar content for the *jand* with maximum sensory score were 11 ppm, 8.89%, 831 g/100 L, 4.97 g/100 L, 68.5 ppm,14.25°Bx, 0.84%, 3.8 and 0.265% respectively *Jand* made at different pHs, levels of *murcha,* and fermentation durations differed significantly ($p<0.05$) with respect to sensory properties. Thus, the findings suggest that it is possible to produce a good quality *jand* by maintaining pH level of 6, fermentation duration of 21 days and *murcha* level of 0.2%.

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Appendixes

Appendix A

Specimen card for sensory evaluation

Hedonic rating test

Name of panelist: **Date:**

Product: ***Bhati 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 parameter.

Perceptions	Points
Like extremely	5
Like slightly	4
Neither like nor dislike	3
Dislike slightly	2
Dislike extremely	1

Appendix B

Variate: Appearance

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Samples	14	13.9600	0.9971	2.77	0.001
Residual	135	48.6000	0.3600		
Total	149	62.5600			

Variate: Smell

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Samples	14	32.5600	2.3257	11.54	<.001
Residual	135	27.2000	0.2015		
Total	149	59.7600			

Variate: Sourness

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Samples	14	58.3733	4.1695	13.97	<.001
Residual	135	40.3000	0.2985		
Total	149	98.6733			

Variate: Taste

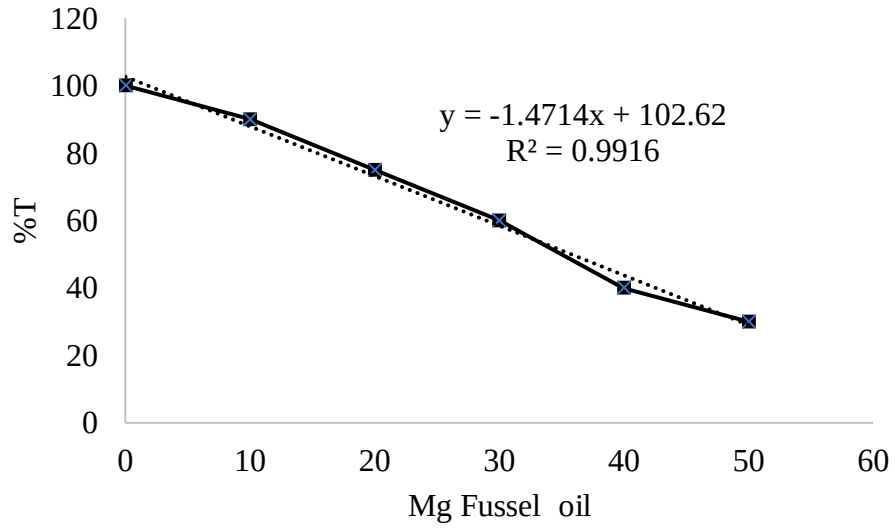
Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Samples	14	61.7600	4.4114	14.49	<.001
Residual	135	41.1000	0.3044		
Total	149	102.8600			

Variate: Overall

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Samples	14	38.7733	2.7695	8.76	<.001
Residual	135	42.7000	0.3163		
Total	149	81.4733			

Appendix-C

Standard curve for higher alcohol



Appendix-D

t-Test: Paired Two Sample for Means ethanol		
	8.89	*6.98*
Mean	8.9	6.55
Variance	0.02	0.005
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
df	1	
t Stat	15.66667	
P(T<=t) one-tail	0.02029	
t Critical one-tail	6.313752	
p(T<=t) two-tail	0.04058	
t Critical two-tail	12.7062	

t-Test: Paired Two Sample for Means aldehyde		
	4.95	*3.15*
Mean	4.99	3.15
Variance	0.0002	0.0008
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
df	1	
t Stat	184	
P(T<=t) one-tail	0.00173	
t Critical one-tail	6.313752	
P (T<=t) two-tail	0.00346	
t Critical two-tail	12.7062	

t-Test: Paired Two Sample for Means of methanol		
	10.5	47.0297
Mean	11.25	47.16
Variance	0.125	0.0392
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
df	1	
t Stat	-92.076923	
P(T<=t) one-tail	0.00345686	
t Critical one-tail	6.31375151	
P(T<=t) two-tail	0.00691373	
t Critical two-tail	12.7062047	

t-Test: Paired Two Sample for higher alcohol

	68.5	69.06
Mean	68.5	69.06
Variance	0.08	0.0008
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
df	1	
t Stat	-3.11111	
P(T<=t) one-tail	0.098994	
t Critical one-tail	6.313752	
P(T<=t) two-tail	0.197988	
t Critical two-tail	12.7062	

t-Test: Paired Two Sample for Means acidity	*0.84*	*0.62*
Mean	0.84	0.615
Variance	0.0008	0.00045
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
df	1	
t Stat	45	
P(T<=t) one-tail	0.007072389	
t Critical one-tail	6.313751515	
P(T<=t) two-tail	0.014144778	
t Critical two-tail	12.70620474	

t- Test: Paired TWO Sample for Means pH	*3.8*	*3.9*	
Mean	3.8	3.75	
Variance	0.02	0.005	
Observations	2	2	
Pearson Correlation	-1		
Hypothesized Mean Difference	0		
df	1		
t Stat	0.33333		
P(T<=t) one-tail	0.39758		
t Critical one-tail	6.31375		
P(T<=t) two-tail	0.79517		no sig
t Critical two-tail	12.7062		

t- Test: Paired TWO Sample for Means TSS	*14.1*	*16.1*
Mean	14.25	16.1
Variance	0.005	0.02
Observations	2	2
Pearson Correlation	1	
Hypothesized Mean Difference	0	
df	1	
t Stat	-37	
P(T<=t) one-tail	0.008601	
t Critical one-tail	6.313752	
P(T<=t) two-tail	0.017202	
t Critical two-tail	12.7062	

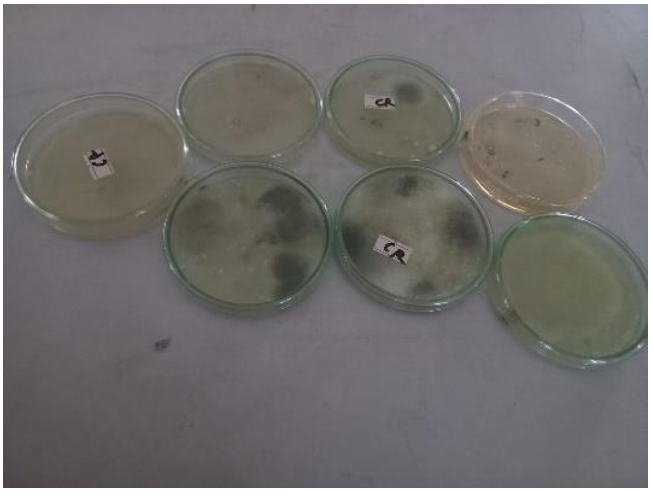
t-Test: Paired Two Sample for Means for reducing sugar		
	0.29	*0.58*
Mean	0.265	0.58
Variance	0.00245	0.0008
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
df	1	
t Stat	-5.72727	
P(T<=t) one-tail	0.055023	
t Critical one-tail	6.313752	
P(T<=t) two-tail	0.110046	
t Critical two-tail	12.7062	

t-Test: Paired Two Sample for Means ester		
	832.09	*533.9*
Mean	831.95	533.3
Variance	0.005	0.02
Observations	2	2
Pearson Correlation	-1	
Hypothesized Mean Difference	0	
df	1	
t Stat	1991	
P(T<=t) one-tail	0.00016	
t Critical one-tail	6.313752	
P(T<=t) two-tail	0.00032	
t Critical two-tail	12.7062	

Color plate

Color plate .1; - *Murcha* collection from local market

Color plate .2; - Microbial analysis



Color plate .3; - Microbial analysis

Color plate .4; - Adjusting water pH

Color plate .5 Higher alcohol evaluation

Color plate .6 Methanol evaluation

Color plate .7; - *Murcha* addition to cooked rice

Color plate .8; - Samples of *Bhati Jand*

Color plate

Color plate .9; - Sensory analysis by panelist

Color plate .10 Sensory analysis by panelist

Color plate .11; - *Murcha* collection from local market

Color plate .12 distillation of *jand*