STUDY ON CHEMICAL PARAMETERS AND CYANOGENS CONTENT OF CASSAVA *(Manihot esculenta* Crantz*) JAND* DURING TRADITIONAL FERMENTATION.

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2018

Study on Chemical Parameters and Cyanogens Content of Cassava *(Manihot esculenta* Crantz*) Jand* During Traditional Fermentation.

A dissertation submitted to the Department of Food Technology, Central Campus of Technology, Tribhuvan University, in partial fulfilment of the requirements for the degree of B. Tech. in Food Technology

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March, 2018

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Approval Letter

This *dissertation* entitled *Study on Chemical Parameters and Cyanogens Content of Cassava (Manihot esculenta* Crantz*) Jand During Traditional Fermentation* presented by Samip Khadka has been accepted as the partial fulfillment of the requirements for the B.Tech. degree in Food Technology.

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Acknowledgements

I would like to express deep gratitude to my guide Mrs Babita Adhikari Assistant Professor, CCT, Hattisar, Dharan for his enthusiastic support and immense suggestion during the course of dissertation work.

I am also indebted to Prof. Dr. Dhan Bahadur Karki, Campus Chief, Central Campus of Technology, Hattisar, Dharan, for his kind support and cooperation throughout the dissertation period. Similarly, I am also grateful to *Assoc*. Prof. Basanta Kumar Rai, Chairperson, Department of Food Technology, CCT, Hattisar, Dharan.

I express thanks to all the teachers and staff members, librarian for their support. My special thanks goes to Mr. Prajwal Bhandari, Head Technical Assistant, CCT, Hattisar, Dharan. Many- many thanks goes to my classmates especially Ramesh Koirala, Samel Pandey, Ashish Niroula, Pragatee Adhikari, Lila Mani Pokhrel and Bimala Shrestha for helping with great effort. Also, I would like to thank my juniors Ajit Kumar Shah, Pankaj Dahal, and Prajwol Pokhrel for their help.

Above all, I am indebted to my parents without whose constant encouragements, inspirations and affection, I would never have reached this far, and this work would have never seen the light of the day.

March, 2018

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Abstract

A study was conducted to know the effect of traditional Nepalese fermentation process of making *jand* on various chemical attributes including the most common group of toxins i.e. cyanogenic glycosides of cassava (*Manihot esculenta* Crantz) tubers. Cassava tubers were bought from the local market of Dharan. Proximate analysis of cassava tubers were carried out. Before the tubers were subjected to fermentation, they were thoroughly cleaned, cut into suitable sizes and cooked.. Inocolum of traditional starter i.e. *murcha* was sprinkled over the cooked tubers and were allowed to ferment in small plastic jars.

Analysis of changes in different parameters i.e. pH, alcohol, TSS, reducing sugars, proteins and cyanogens were analysed in the regular interval of 3 days until the fermentation was complete. Alcohol content of the mash increased gradually from nil to 12.403 % m/v during initial 12 days of fermentation and finally reached upto 13.6 m/v in 21st day of fermentation. TSS of brew was found to be increasing initially where it reached 16.47ºBx in 9th day of fermentation and finally decreased to 7 upto the final 21st day of fermentation. Initially not traceable amount of reducing sugars in tubers reached upto 11.87 % in 6th day of fermentation and was recorded 0.57% in 12th day after which it wasn't detected by Lane and Eynon method. Initial pH of cassava was found to be 6.11 which dropped to the final pH of 3.64% in final product. Final protein content of product was analysed to be 3.195% from the initial value of 1.84%. Initial cyanogens content of 75.124 mg HCN/kg fresh wt of cassava was reduced to 45.17 mg HCN/kg after boiling which was analysed to be just 6.911mg HCN/kg in final product which was below the marked permitable limit for cyanogens recommended by FAO. A sheer drop of 90.8% in cyanogens contents of cassava was seen during the overall process of *jand* preparation including both boiling and fermentation.

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List of Abbreviations

Abbreviations	Full Form
^{o}Bx	Degree Brix
^{o}C	Centigrade
ADP	Adenosine Diphosphate
ANOVA	Analysis of Variance
AOAC	Association of Analytical Chemists
ATP	Adenosine Triphosphate
CCT	Central Campus of Technology
dL	Decaliter
EMP	Embden-Meyerhof Pathway
FAO	Food and Agriculture Organization
LAB	Lactic Acid Bacteria
TCA	Tricarboxylic acid cycle

PART I

Introduction

1.1 General introduction

Most food and beverage fermentation processes are ancient rituals that humans have been performing since before the dawn of history. Food fermentation is regarded as one of the oldest methods of food processing and preservation. Techniques evolved by disparate human cultures over millennia, through observation of natural phenomena and manipulating conditions with trial and error. 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 the wide range of fermented foods and beverages contributed significantly to the diet of many people (Achi, 2005; Katz, 2012). Fermented foods enjoy worldwide popularity as attractive, wholesome and nutritious components of our diet. Fermented foods have always been generally regarded as safe, but this reputation has been seriously threatened in recent years by incidents such as outbreaks of illness caused by pathogens in various fermented food products (Nout, 2001).

Alcoholic beverages are produced primarily from cereals, fruits and tubers. Cereal based alcoholic beverages, viz *jand*, *toongba* and *nigar* have a very long tradition in Nepal. The common cereal of choice for *jand* preparation is finger millet but other cereals like maize, wheat and rice are also used (Rai, 1991).

Murcha is a mixed starter culture used in the Preparation of alcoholic beverages like *jand*. It is a ball like starter, used to ferment starchy materials into fermented beverages in Nepal, Darjeeling hills and Sikkim (Tamang, 2010). *Murcha* contains saccharifying molds, lactic acid bacteria and fermentating yeast and is therefore the result of concerted action of these microorganisms on the cooked cereals (KC *et al.*, 2004). *Jand* is traditionally prepared alcoholic beverages (Kharel *et al*., 2007). It is served in different forms and

modes. Strained *jand* is prepared by leaching out the readily extractable contents from the mash with lukewarm water. The beverage is cloudy in appearance and has a very short shelf-life. The shelf life of strained *jand* can be extended to a few months by in-bottle 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).

Cassava *jand* is a traditional fermented alcoholic beverage prepared and consumed by *Rai* and *Limbu* communities, especially in districts of eastern Nepal like Khotang, Udayapur, Dhankuta, Sunsari, Jhapa and Ilam. Among the communities cassava *jand* is one of the socially accepted alcoholic beverages. It is turbid thick, cloudy in appearance.

1.2 Statement of the problem

Cassava is one of the major energy source for people living in various tropical and sub tropical regions of the world. In context of Nepal, though underutilized, it is widely cultivated and consumed in some regions. Besides of being energy rich, cassava also contains some components, mainly cyanogens which may turn out to be fatal if consumed in high amount or if under processed cassava is consumed. In eastern region of Nepal there is trend of fermenting cassava to prepare *jand* and it is highly praised and widely consumed as well. Generally fermentation is believed to have increasing effects on overall nutritive value of any food. But in cases of foods like cassava which contains potentially lethal toxins, study of effects of fermentation on overall nutritive value and toxin level would give a clear idea whether to consider such foods safe or not and also helps to devise an optimum processing method which ensures high nutritive value with least toxic effects.

1.3 Objectives

1.3.1 General objective

The general objective of this study is to observe the effects of traditional Nepalese alcoholic fermentation on the nutritional and toxic components of cassava.

1.3.2 Specific objectives

The specific objectives of the study are as follows:

1. To prepare Cassava *jand* and to carry out its analysis.

2. To study the effect of traditional Nepalese alcoholic fermentation process on the cyanogenic glycoside content of cassava tubers.

3. To study the chemical changes occurring during the course of fermentation.

1.4 Significance of study

With the increasing population there is sheer rise in the demand of highly nutritious and high energy giving food in the entire world. Cassava which is primarily grown in less arable, densely populated regions of tropics and sub tropics can be a better alternative for ever increasing food demand. Cassava is cultivated and processed in different ways in different regions of the world. However, due to illiteracy and lack of proper knowledge people barely pay attention to the nutritive as well as toxic aspects of cassava. If properly processed, this carbohydrate rich tuber could be the answer to the food insufficiency faced by a large population of the world but sometimes due to poor processing techniques it may be categorised as the life threatening factor.

Cassava is a food crop that is cultivated in Nepal also and consumed in different forms. In eastern regions of Nepal a tradition of fermenting cassava to prepare the *jand* is widely practiced and is consumed with great appreciation for its taste. However, no intense studies have been carried out to observe the nutritional and toxic effects of cassava *jand*. The results of this research could help the local people clarify their view regarding not only the safety of cassava *jand* but also the positive nutritional aspects. Also it could be the basis of promoting the indigenous food to international markets and thus could help carry the identity of our ethnic communities, tribes and nation to every corner of the world. Also it will encourage the farmers to produce more cassava for this kind of value added products which will then help to provide better price for their hard work.

1.5 Limitation of the study

During the dissertation work, the following limitations were taken into account:

1. Single variety of cassava was studied.

2. The detailed chemical composition of *jand* prepared could not be carried out due to lack of time and limited resources.

3. Activity of enzymes was not studied.

4. Strain and its concentration were not controlled.

PART II

Literature review

2.1 Cassava

The cassava (*Manihot esculenta* Crantz) is cultivated mainly in the tropic and sub-tropic regions of the world, over a wide range of environmental and soil conditions. It is very tolerant of drought and heat stress and produces well on marginal soils. It is an important dietary staple in many countries within the tropical regions of the world (Perez and Villamayor, 1984) where it provides food for more than 800 million people (FAO, 2007). As a subsistence crop, cassava is the third most important carbohydrate food source in the tropics after rice and maize, providing more than 60% of the daily calorific needs of the populations in tropical Africa and Central America(Nartey, 1978). According to Alexandratos (1995), cassava plays an important role in alleviating food problems, because it thrives and produces stable yields under conditions in which other crops fail.

Kingdom	Plantae
Subkingdom	Tracheobionta
Superdivision	Spermatophyta
Division	Magnoliophyta
Class	Magnoliopsida
Subclass	Rosidae
Order	Euphorbiales
Family	Euphorbiaceae
Genus	Manihot
Species	*Manihot esculenta* Crantz

Source: USDA, (2017)

Cassava is a versatile crop and can be processed into a wide range of products such as starch, flour, tapioca, beverages and cassava chips for animal feed. Cassava is also gaining

prominence as an important crop for the emerging biofuel industry and, as corroborated by Ziska *et al.* (2009), is a potential carbohydrate source for ethanol production. A well planned strategy for the development and utilisation of cassava and cassava products can provide incentives for farmers, crop vendors and food processors to increase their incomes. It can also provide food security for households producing and consuming cassava and cassava products (Plucknett *et al.*, 1998).

Traditionally, cassava has been grown by farmers throughout the Bahama Islands and has been of particular importance to small farmers of the central and southeastern islands, where it is still cultivated. It is a crop that is generally grown on marginal lands with a minimum of agricultural 2 inputs (Hillocks *et al.*, 2002). Once established, the cassava crop is given little attention, but still is able to tolerate weed competition, as well as insect pests and diseases. The potential exists for improving the productivity of cassava through better agronomic practices, superior varieties and pest and disease management.

History of cassava in Nepal dates back to early 60s. Among five Developmental Regions (DR), the area under this crop is the highest in the Eastern DR and the lowest in the Far-Western DR. In most places, the production techniques are local and traditional and the yield appears to be low. The crop has not been accorded with any official attention and, hence, no organized research has been conducted to develop the crop. Although some local methods of cassava utilization exist, processing units on a commercial scale are absent (Shrestha, 1992).

2.1.1 Agronomy of cassava

Cassava is a tropical root crop, requiring at least 8 months of warm weather to produce a crop. It can be grown in extremes of rainfall. In most of regions, it doesn't tolerate flooding. In droughty areas it loses its leaves to conserve moisture, producing new leaves when rains resume. It takes 18 or more months to produce a crop under adverse conditions such as cool or dry weather. Cassava does not tolerate freezing conditions and produce poor yield. It tolerates a wide range of soil pH 4.5 to 8 and is most productive in full sun. Cassava is mostly propagated vegetatively (stem cuttings) by planting a piece of the stalk, which grows into a new plant (Agrifarming, 2017).

Cassava is mainly grown in tropical areas and it holds an important place as traditional and staple food of various African least developed nations. Besides them south east Asian

countries contribute more in global production of cassava. In 2014, 268 million tons of cassava was produced. The world's largest producer of cassava is Nigeria with a production of 47,406,770 tons in 2013. With a production of 30,227,542 tons, Thailand follows next. Indonesia (23,936,920) and Brazil (21,484,218) are ranked third and fourth in the world in cassava production. Top ten countries in terms of production of cassava are listed in Table 2.1.

Table 2.1 Top 10 cassava producing countries in the world

Rank	Country	Production value of cassava (in tons)
1	Nigeria	47,406,770
2	Thailand	30,227,542
3	Indonesia	23,936,920
4	Brazil	21,484,218
5	Angola	16,411,674
6	Ghana	15,989,940
7	Democratic Republic of Congo	14,611,911
8	Vietnam	9,757,681
9	Cambodia	7,572,344
10	India	7,236,600

Source: Worldatlas, (2017)

2.1.2 Chemical and nutritional composition of cassava roots

Cassava roots and cassava leaves are used are used for human consumption and animal feed. Cassava roots are rich in digestible carbohydrates, mainly in starch. Cassava root starch consists of both amylase (20%) and amylopectin (70%) (Buitrago, 1990). There is a large variation in sucrose content between cassava genotypes. In sweet varieties, sucrose

constitutes about 17% of total carbohydrates (Hendershott, 1972). Generally, cassava roots have less than 1% free sugars. Cassava roots are low in protein and fat. Cassava roots have less than the recommended minimum limit in almost all essential amino acids, except tryptophan. Cassava roots should be eaten along with other crops rich in essential amino acids to supplement the deficit, such as vegetables, cereals, fish and meat. Cassava leaves are much richer in protein than the roots, although the leaf contains a lower proportion of methionine than the root protein. The levels of all other essential amino acids in leaf portion exceed the FAO's reference. Cassava has a high content of dietary fibre, magnesium, sodium, riboflavin, thiamine, nicotinic acid and the citrate (J. H. Bradbury and Holloway, 1988). Proximate chemical composition as described by USDA is tabulated in Table 2.2.

Table 2.2 Chemical composition of cassava

Nutrient proximate	Value per 100 g	1 root 480 g
Water	59.68	243.49
Energy	160	653
Protein	1.36	5.55
Total lipid (fat)	0.28	1.14
Carbohydrates, by	38.06	155.28
Fiber, total dietary	1.8	7.3
Sugars, total	1.7	6.94

Source: USDA, (2016)

2.1.3 Cassava and cyanogenic glycosides

The cyanogenic glycosides belong to the products of secondary metabolism, to the natural products of plants. These compounds are composed of an a-hydroxynitrile type aglycone and of a sugar moiety (mostly D-glucose). The distribution of the cyanogenic glycosides (CGs) in the plant kingdom is relatively wide, the number of CG-containing taxa is at least

2500, and a lot of such taxa belong to families Fabaceae, Rosaceae, Linaceae, Compositae and others (Vetter, 2000).

Cassava is the third most important food source in the tropics after rice and maize and is the staple food of at least 500 million people (Cock, 1985). Cassava is easy to grow, yields well in good conditions and even in poor soils subject to dry conditions it still produces edible roots. The roots are very starchy and the young leaves are a good source of protein (Bradbury and Holloway, 1988). Because of the perceived agricultural advantages of growing cassava and increasing population pressures its usage is being extended to regions in Africa and elsewhere in which it was not formerly used.

As a defence mechanism against attack by predators, cassava produces two cyanogenic glucosides; linamarin and a small amount of lotaustralin (methyl linamarin). These cyanogens are distributed widely throughout the plant, with large amounts in the leaves and the root cortex (skin layer), and generally smaller amounts in the root parenchyma (interior). In so-called sweet cassava the parenchyma contains only a small amount of cyanogens, so that after peeling, these roots can be safely boiled and eaten, as occurs in the South Pacific (Bradbury and Holloway, 1988). The bitter taste of bitter cassava is very largely due to linamarin (King and Bradbury, 1995) and high cyanide parenchyma roots must be processed before consumption to reduce the amount of toxic cyanogens to a safe level. The World Health Organisation (WHO) has set the safe level of cyanogens in cassava flour at 10ppm (FAO, 1991), and the acceptable limit in Indonesia is 40 ppm (Damardjati *et al.*, 1993; Djazuli and Bradbury, 1999).

2.1.3.1 Cyanogenesis in cassava

The cyanogenic glycosides are a group of nitrile-containing plant secondary compounds that yield cyanide (cyanogenesis) following their enzymatic breakdown. The functions of cyanogenic glycosides remain to be determined in many plants; however, in some plants they have been implicated as herbivore deterrents and as transportable forms of reduced nitrogen. It is estimated that between 3,000 and 12,000 plant species produce and sequester cyanogenic glycosides, including many important crop species such as sorghum, almonds, lima beans (non-domesticated), and white clover. The most agronomically important of the cyanogenic crops, however, is the tropical root crop cassava (*Manihot esculenta* Crantz). More than 153 million tons of cassava are produced annually, and it is the major source of

calories for many people living in the tropics, particularly sub-Saharan Africa (White *et al.*, 1998).

Cyanogenesis is initiated in cassava when the plant tissue is damaged. Rupture of the vacuole releases linamarin (Fig. 2.2), which is hydrolyzed by linamarase, a cell wall-associated β-glycosidase (McMahon *et al.*, 1995). Hydrolysis of linamarin yields an unstable hydroxynitrile intermediate, acetone cyanohydrin, plus Glc. Acetone cyanohydrin spontaneously decomposes to acetone and HCN at pH 5.0 or temperatures 35°C and can be broken down enzymatically by HNL (Hasslacher *et al.*, 1996; Wajant and Pfizenmaier, 1996).

The primary cyanogenic glycosides present in cassava are linamarin and which accounts for the 90% of total cyanogenic glycosides in cassava and rest 10% is credited to lotaustralin (Fig. 2.1). The first step in the conversion of linamarin to cyanide is the deglycosylation or hydrolysis of linamarin by linamarase to form acetone cyanohydrins and glucose. Since acetone cyanohydrins may spontaneously or enzymatically decompose to cyanide and acetone, it has generally been assumed that linamarase activity is the rate limiting step in cyanogenesis (Fig. 2.3). The final step of cyanogenesis from linamarin is the breakdown of acetone cyanohydrins to cyanide and acetone. This reaction can occur spontaneously at temperatures greater than 35ºC or at pH greater than 4.0. Acetone cyanohydrin can also be converted to cyanide and acetone by hydroxyl nitrile lyase (HNL). Both the spontaneous and the enzyme catalysed decomposition of acetone cyanohydrin affect the cyanogens content of cassava food products (McMahon *et al.*, 1995)

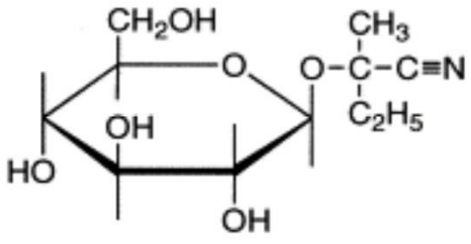

Fig. 2.1 Structure of lotaustralin

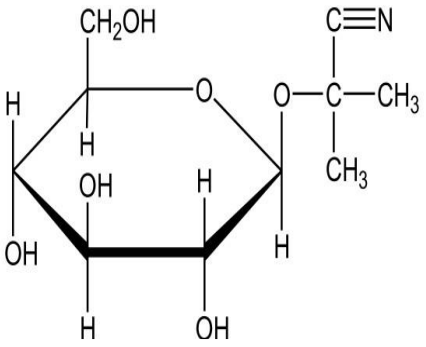

Fig. 2.2 Structure of Linamarin

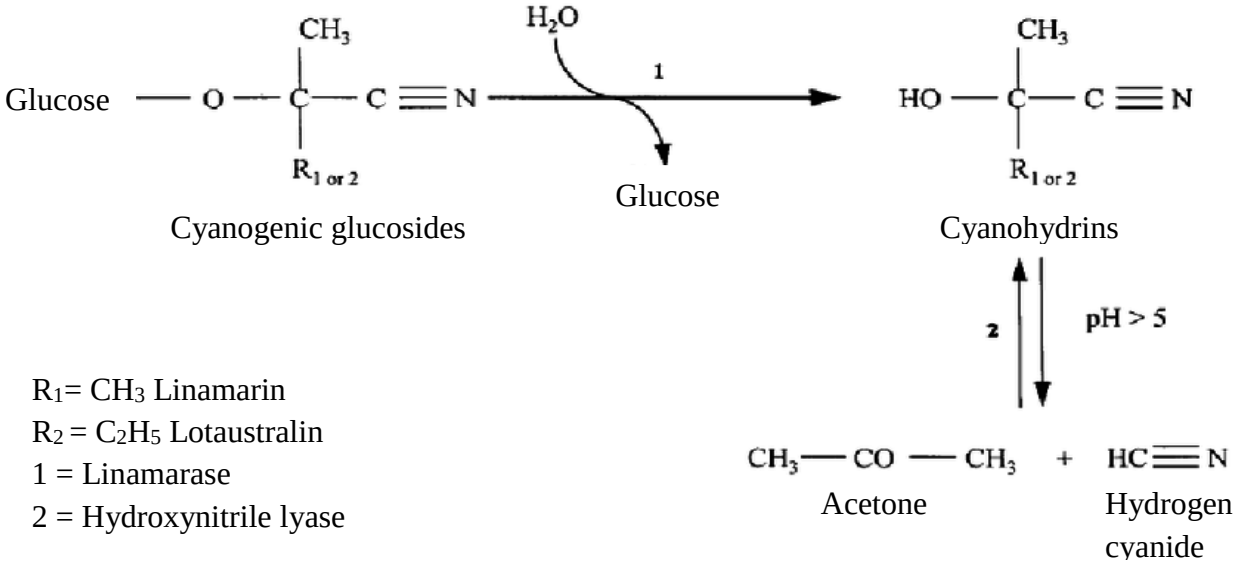

Fig. 2.3 Cyanogenesis in cassava

Source: Kasi *et al.*, (2014)

2.1.3.2 Cyanogen toxicity

Africa produces more than 30 million tons of cassava on about 5 million hectares (6 tons per hectare). Approximately 80% of the root production and 70% of the harvested area are from Western Africa. Recent reports suggest that the ingestion of poorly processed cassava roots is associated with the incidence of an ataxic neuropathy (konzo) in African countries. When cassava-based diets are not supplemented with good sources of protein and iodine, goiter and rickets are also prevalent. In certain countries of Africa where the rate of ataxic neuropathy is high, the incidence of thyroid disorders is also high. Persons consuming poorly processed cassava in large quantities are susceptible to neuropathologies caused by cyanide. Cyanide detoxification in the body is impaired by protein deficiency. Cyanide is very poisonous because it binds to an enzyme called cytochrome oxidase and stops its action in respiration, which is the key energy conversion process in the body. The lethal dose of cyanide for an adult depends on body weight and is between 30 and 210 mg of hydrogen cyanide. Sometimes these limits are exceeded by persons eating a cassava meal and deaths occur due to cyanide poisoning. Smaller (non-fatal) amounts of cyanide cause acute intoxication with symptoms of dizziness, headache, nausea, stomach pains, vomiting and diarrhea.When properly processed, the root of cassava is safe and cheap as a major dietary energy source for humans and domestic animals; however, a cassava-based diet

will lack sufficient protein and will be particularly deficient for the growth and development of children unless it is supplemented by protein from animal, including fish, or legume sources. Cassava leaves, if they are appropriately cooked, can be a useful source of some nutrients (Teles, 2002).

On 14 January 2008, the Centre for Food Safety (CFS) advised members of the public to avoid consuming Piranha brand crackers and snacks manufactured by Tixana Australia Pty Ltd. The appeal was made following a warning issued by the Food Standards Australia New Zealand (FSANZ) due to the higher-than-usual levels of naturally occurring cyanogenic glycosides in the ingredient cassava in a batch of exported vegetable crackers. The CFS contacted the relevant authorities and was informed that the affected products had been exported to Hong Kong. The CFS alerted the trade to stop selling the affected products (Kwok, 2008).

Consumption of cassava and its products that contain large amounts of cyanogens may cause cyanide poisoning with symptoms of vomiting, nausea, dizziness, stomach pains, weakness, headache and diarrhoea and occasionally death (Akintonwa *et al.*, 1995; Mlingi *et al.*, 1992). Cyanide intake from cassava exacerbates goitre and cretinism in iodine deficient areas (Delange *et al.*, 1994) and is almost certainly the cause of konzo in eastern, central and southern Africa. Konzo is an irreversible paralysis of the legs of sudden onset, which occurs particularly in children and women of child bearing age (Cliff *et al.*, 1997; Howlett *et al.*, 1990). Tropical ataxic neuropathy (TAN) is a chronic condition of gradual onset that occurs in older people who consume a monotonous cassava diet. It causes loss of vision, ataxia of gait, deafness and weakness (Howlett, 1994; Onabolu *et al.*, 2001; Osuntokun, 1994).

2.1.4 Other anti-nutritional factors present in cassava

When it comes to cassava, cyanogens are considered to be the most prominent limiting factor that causes the consumption of cassava unsafe. But besides them, cassava also constitutes of some of the anti nutritional factors that bind with the various nutritional components and make them sparsely available for the consumers. Major anti-nutritional factors present in cassava are briefly discussed as follows.

2.1.4.1 Phytate

Phytate (inositol hexakisphosphate) is a regulator of intracellular signaling and a form of phosphate storage in plant seeds, but it can bind proteins and minerals in the gastrointestinal tract preventing absorption and utilization by the body. Specifically, phytate interferes with the absorption of divalent metals, such as iron and zinc, which are essential nutrients (Hambidge *et al.*, 2008).

Various studies show that the cassava varieties have only minute amount of phytate content, which can be reduced by cooking and fermentation methods. Haritha and Jayadev (2017) have mentioned the phytate content to be 53.7 mg/100 g for sweet varieties and 62.4 mg/100 g for bitter varieties of cassava.

2.1.4.2 Oxalate

Oxalates are anti-nutrients that negatively affect calcium and magnesium bioavailability (Massey, 2007). It can bind calcium and be excreted through the urine or form crystals. Calcium oxalate crystals are a major constituent of kidney stones and in people who are susceptible, increasing calcium and decreasing oxalate intakes are advised. The oxalate content in cassava is low which again becomes negligible on processing. Haritha and Jayadev (2017) have reported the oxalate content of cassava to be 1.30 mg/100 g for sweet and 3.27 mg/100 g for bitter varieties of cassava.

2.1.4.3 Tannins

Tannins have traditionally been considered anti-nutritional, but it is now known that their beneficial or anti-nutritional properties depends upon their chemical structures and dosage(Muller and McAllan, 1992). Tannins content decreases on boiling of cassava roots. The study of anti-nutritional parameters conducted in a study shows that the tannin in the bitter cassava (0.60±0.22 mg/100 g) is higher than that of the sweet variety (0.40±0.01 mg/100 g) (Haritha and Jayadev, 2017).

2.2 Historical background of alcoholic beverage

Alcoholic beverages are believed to have originated in Egypt and Mesopotamia some 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 microorganisms to stimulate the biochemical changes was demonstrated several years later. Alcoholic fermentation was first identified by Gay Lussac in 1810, but at that time yeast was not recognized as causative organism. Schwan in 1835 demonstrated that yeast could produce alcohol and carbon dioxide when introduced in sugar-containing solution. He termed yeast *Zuckerpilz* meaning sugar-fungus, from which the name *Saccharomyces* originated (Prescott *et al.*, 1987). *Saccharomyces* group possesses almost all the credit of producing alcoholic beverages.

The production and consumption of alcoholic beverage is one of the man's oldest activities. Today brewing, wine making and distilling are of major commercial importance in many non-Islamic countries and, through taxation, can be an important source of government revenue (Varnam and Sutherland, 1994). In Nepal, the technique of alcoholic beverage production remains largely traditional. The method has been practiced from very ancient time but the exact principle involved in the process is not yet known. However, it is widely in use and it is difficult to say when and from where the technology had come to Nepal. It is said that the custom of worshipping Gods and Goddess was by *Tantric* process and alcoholic beverage were offered during worship (Karki, 1986) .

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 cellfree yeast juice, which led to the discovery of the role of enzymes in fermentation. He called the enzyme "Zymase". 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 (Barnett, 2005).

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 (Bokulich, 2017)

2.3 Organisms responsible for food fermentation

Fermented foods, a part of important food eco system, harness a microbial diversity and converse the functional organisms in the environment. Filamentous molds, yeasts and bacteria constitute the organisms present in fermented foods and beverages.

2.3.1 Bacteria

Several bacterial families are present in foods, the majority of which are concerned with food spoilage. As a result, the important role of bacteria in the food fermentation is often overlooked. However, a number of bacteria find use in fermented industries. The most important bacteria in desirable food fermentation are the Lactobacillaceae which have the ability to produce lactic acid from carbohydrates. Other important bacteria, especially in the fermentation of fruits and vegetables, are the acetic acid producing *Acetobacter* species (FAO, 1998)

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 genus *Bacillus* in alkaline fermentations. Sometimes, a mixture of these organisms can be used to prepare special products (Steinkraus, 1996)

2.3.1.1 Lactic acid bacteria

The lactic acid bacteria (LAB) are rod shaped bacilli ir cocci characterized by an increased tolerance to a lower pH range. This aspect partially enables LAB to outcompete other bacteria in a natural fermentation, as they can withstand the increased acidity from organic acid production (e.g. lactic acid) Sonomoto and Yokota (2011).

LABs are extensively used in the production of fermented milk products, fermented vegetables, pickles, and fermented meat products. They also occur in almost all of the cereal based traditional alcoholic fermentation. Representative homilactic LAB genera include *Lactococcus, Enterococcus, Sterptococcus, Pediococcus* and group I lactobacilli and those heterofermentative LAB include *Leuconostoc, Oenococcus, Weissella*, and group III lactobacilli (Wikipedia, 2017).

2.3.1.2 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 (Heredia *et al.*, 2009), whereas *Bacillus subtilis* is an organism of great significance in oriental soybean fermentations such as *natto* (Wang and Hesseltine, 1987) and *kinema* (Dahal *et al.*, 2005).

2.3.1.3 Molds

Molds are highly aerobic, multicellular eukaryotes, which grow best at an acidic pH and at a temperature of around 25°C. They lack chlorophyll and have mycelial structure, which give the impression of fluffy/cottony colon (Rai, 2009).

Molds play a very important role in oriental food fermentations. They are used in the production of food and beverages ranging from *tempeh, sake* to *jand.* In *sake and jand*, the molds are responsible for saccharifying the starch into simpler 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).

2.3.2.4 Yeasts

Yeasts are a group of unicellular fungi having a cosmopolitan distribution, most of which are represented in sub division *Ascomycotina* and *Basidiomycotina* of the kingdom *Mycota* (KC *et al.*, 2004). Like bacteria and molds, yeasts can have beneficial and non-beneficial effects in foods, and the most beneficial yeasts for desirable food fermentation are *Saccharomyces*, playing roles such as the leavening of bread and the production of alcohol and invert sugar (Marshall and Meija, 2011)

2.4 Fermented foods and beverages

Fermented foods have been defined as those foods which have been subjected to the action of or enzymes so that desirable biochemical changes cause significant modification to the food (Campbell-Platt, 1987). Fermented foods and beverages preparation involves technology from the most primitive to the most advanced, and achieving an astounding range of sensory and textural qualities in the final products. While most of these

fermentations remain at the level of village or household arts, others have achieved massive commercial application and play a significant part in most national economies. All such fermentations have been or remain classified as indigenous, native to a country or culture (Smith, 1996).

2.4.1 Indigenous fermented foods

Indigenous food fermentation is one of the oldest food biotechnological processes dependent on the biological activity of microorganisms (Ross *et al.*, 2002), from which development of fermented foods is achieved in the cultural history of human being (Geisen and Holzapfel, 1996). During the process locally available ingredient(s) either plant or an animal-origin are converted biochemically and organoleptically into upgraded edible products called fermented foods (Steinkraus, 1996).

2.5 Traditional alcoholic beverages

Alcoholic beverages have played an important role in human spiritual and cultural life both in Eastern and Western societies. Unlike in Europe and the Middle East, where indigenous alcoholic beverages are produced primarily from fruit, alcoholic beverages are produced from cereals in the Asia-Pacific region, and serve as an important source of nutrients. European beer uses barley malt as the primary raw material, while Asian beer utilizes rice with molded starters as the raw material. Beverages vary from crystal-clear products to turbid thick gruels and pastes. Clear products which are generally referred to as *shaosingjiu* in China, *chongju* in Korea and *sake* in Japan, contain at least 15% alcohol and are designated as rice-wine, while turbid beverages, such as *takju* in Korea and *tapusy* in the Philippines which contain less than 8% alcohol along with suspended insoluble solids and live yeasts, are referred to as rice-beer (Haard, 1999). Indigenous alcoholic beverages not only add nutrients to the local diet, but also play an important part in the local custom (Karki and Kharel, 2010).

2.5.1 Traditional alcoholic beverages of Nepal

In Nepal, the history of alcoholic beverage dates back to the ancient times. Among the various fermented foods, *jand (chhyang or tongba or poko)* and *raksi* are major alcoholic fermented foods. *Murcha* (yeast) starters are common for the necessary fermentation to

produce these products in Nepal, some parts of India and Southeast China (Tamang and Sarkar, 1995).

2.5.1.1 *Nigar*

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

2.5.1.2 *Raksi*

Raksi (also spelt *rakshi, rukshi*) is an unaged congeneric spirit obtained by pot distillation of the slurry of *jand*. The product is somewhat similar with whiskey and has highly varying alcohol contents (KC *et al.*, 2004), generally between of 15 and 40% (Subba, 1985). Several basic researches have been done on *raksi* production from different cereals using *murcha* as well as pure cultures isolated thereof (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 *rakshi* (Rai, 2006) .

2.5.1.3 *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, 1985) 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). The annual production of *jand* is higher than that of any other indigenous fermented products and that trade is probably the single-most important economic activity among most ethinic groups of low income category (Subba *et al.*, 2005).

Jand is served in different forms and modes. Strained *jand* is prepard by leaching out the readily extractable contents from the mash with luke warm water. The beverage is cloudy in appearance and has a very short shelf life of the order of few hours but 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).

2.5.1.3.1 Types of *jand* as per clarity

In Nepal *jand* are of various types. They can be classified as per the grain used for the production such as rice *jand*, millet *jand*, maize *jand*, wheat *jand*, etc and also as per the clarity of the product (*jand*). As per the clarity *jand* can be classified as strained *jand* and bhatte *jand* or bhyabar (Shrestha and Rai, 2007).

A. Strained *jand*

Strained *jand* is prepared by leaching out the readily extractable contents from the mash with lukewarm water. The beverage is cloudy in appearance and has a very short shelf-life, of the order of few hours (Rai, 1991). A brief outline of *jand* preparation from finger-millet is shown in Fig. 2.4.

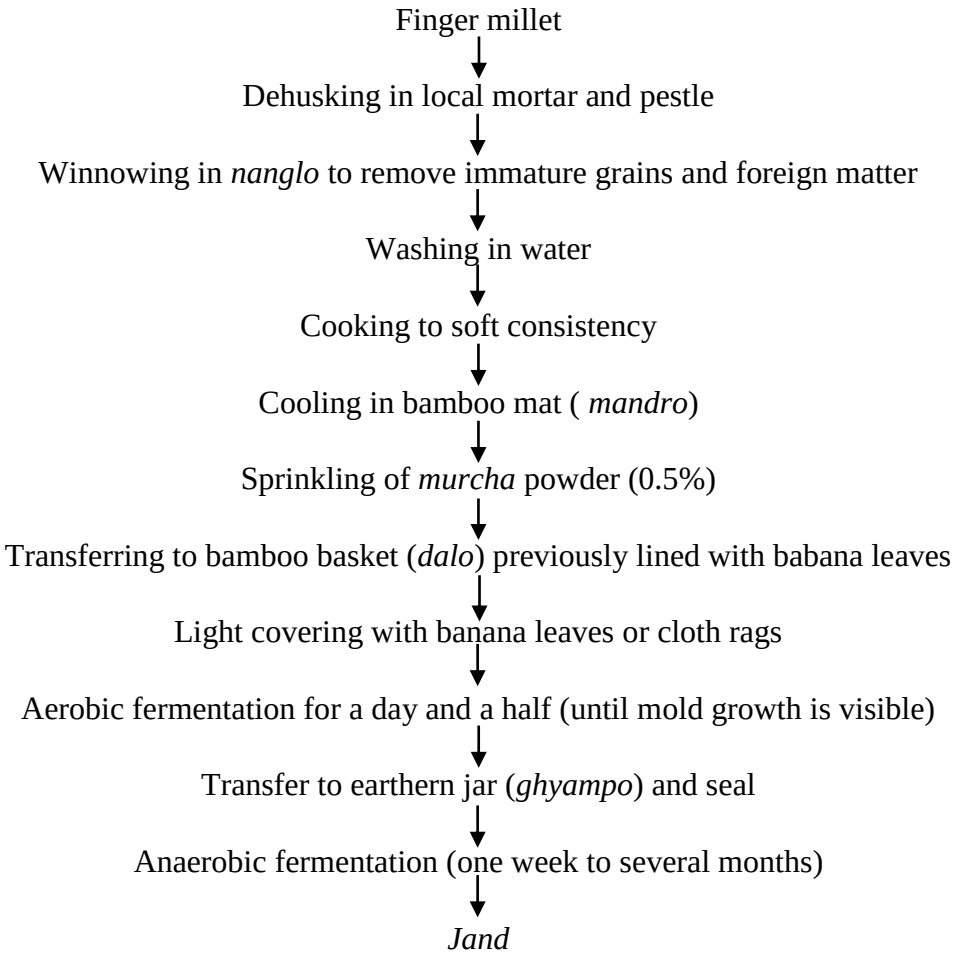

Fig. 2.4 Traditional method of preparing *jand* from finger millet

B. *Bhattejand / bhyabar*

A mixture of the juice and *poko* (fermented solid mass) is known as bhyabar. *Bhayabar* is considered to have the strong intoxicant property (Shrestha and Rai, 2007). Outline of the manufacture to *bhattejand / bhyabar* in east Sikkim, India is shown in Fig. 2.5 (Tamang *et al.*, 1988).

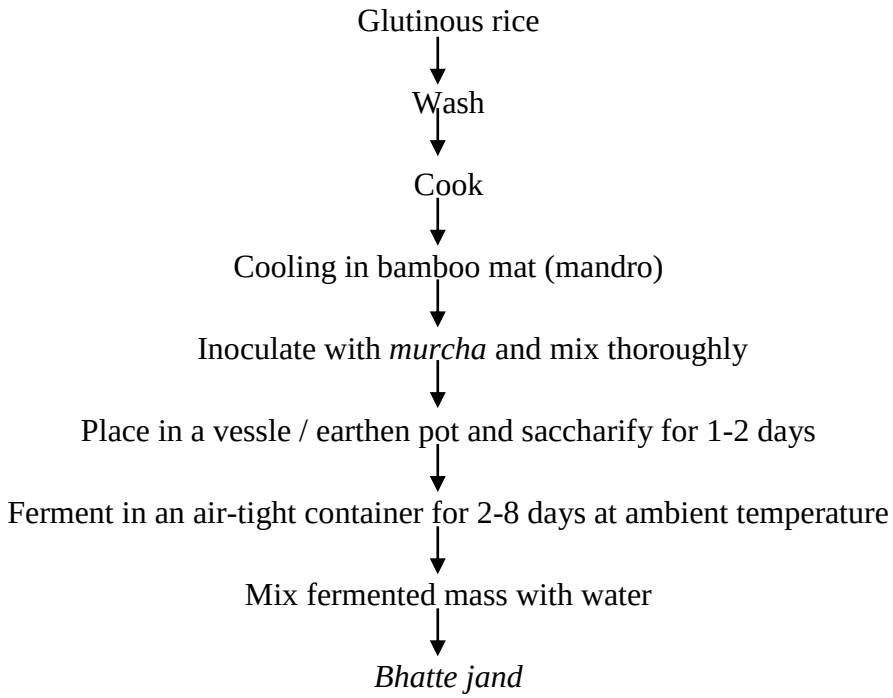

Fig. 2.5 Process of preparing *Bhatte jand*

2.5.1.3.2 Starchy raw materials for the production of *jand* and other 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 *et al.*, 1987). In Nepal, cereal grains (rice, wheat, barley, millet etc.) are used for the production of traditional alcoholic beverages viz. *jand* (undistilled) and rakshi (distilled) using *murcha* as a starter. Although the term *jand* is commonly used in the finger millet beer, beers from maize, rice etc. are also called *jand* the name of the beer is deriving from the raw material used for fermentation e.g. makai *jand* (maize beer), vate *jand* (rice beer) (Tamang *et al.*, 1988). Although various cereal grains are used for the

production of *jand*, the one prepared from finger millet, for various reasons, will possess unmatchable quality.

2.5.1.3.3 Saccharification of starch

Saccharification process is a useful method to convert starchy agricultural materials to sugars that can be utilized as a food ingredient or an essential nutrient source for microbial fermentation. The objective of saccharification is to convert starch to D-glucose as much as possible (Rai, 2009).

2.5.1.3.4 Saccharification process

Starch is liquefied mainly by the action of amylase and amylopectin. Liquefaction is followed by saccharification, a process due to the action of and P-amylases, which yields predominantly maltose with some monnoses, oligosaccharides, and dextrins (Prescott *et al.*, 1987). The amylose and amylopectin of cereal starch are converted by a-amylase during liquefaction to a collection of linear and branched dextrins. The linear dextrins are rapidly and almost totally converted to D-glucose by glucoamylase. The branched dextrins are much less susceptible to hydrolysis owing to the lower rate at which glucoamylase cleavage the alpha (l,6) D-glucosidic linkages as compared to cleavage of the alpha (1,4) D-glucosidic linkage. For practical purposes, the dextrin hydrolysis reactions are irreversible. However, hydrolysis to D-glucose is not complete because simultaneous condensation reactions occur where in D-glucose is condensed to reversion problem. By using glucoamylase, it is possible to convert starch almost totally (99%) to D-glucose, but economically it is not feasible (Verma, 1991).

2.5.1.3.5 Methods of saccharification

A. Malt enzyme

The first stage in the preparation of spirits, such as whiskey, from starch material is the conversion of starch into a dextrin-maltose mixture with intermediates. This is aeconnplished, as in the brewing industry, by the action of a diasticenyme such as from malted grain (Pederson, 1971),

B. Mold bran

Mold bran is an enzynne product obtained by growing *Aspergillus oryzae* or other mold on moist, sterilized bran. It is and may be used successfully as a substitute for malt in the saccharification of grain, potato or other types of starch containing mashes. Smaller quantities of mold bran than the malt are required to saccharify a given quantity of grain mash (Prescott and Dunn, 1959).

Hao, fulmer and Underkofler studied mold bran prepared from 37 strains of mold of genera *Aspergillus*, *Mucor*, *Penicillium* and *Rhizopus*. They found that bran preparation made with strain of *A. oryzae, R. delemar* and *R. oryzae* were optimum for the saccharification of corn mashes as evaluated by the yield of ethanol produced. Strain of *A. oryzae* were selected as being best suited for industrial use, based on ease of handling consistency of result and high yield of alcohol from the saccharified mashes, *A. oryzae* ISC 38b has been used in the commercial production of mold bran (Prescott and Dunn, 1959).

C. Mold *koji*

Molds used by the Japanese for many types of food and beverage fermentations are cultured on bulky, fibrous carrier, usually rice, wheat or bran, occasionally barley or soybeans. These mold cultures are termed koji (Steinkraus, 1996). The word koji is an abbreviation of the Japanese word "kabitachi" meaning "bloom of mold". Koji applies to molded masses of cereals, legumes or flours of these seeds which serve as a source of enzymes and in some cases as inocula for larger quantities of non-fermented materials of koji, this refers not to the end product of a traditional fermentation process but rather to the first stage in a process usually involving a second fermentation and is analogous to the use of malt (Tanimura *et al.*, 1978).

2.5.2 Biochemistry of alcohol fermentation by yeasts

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

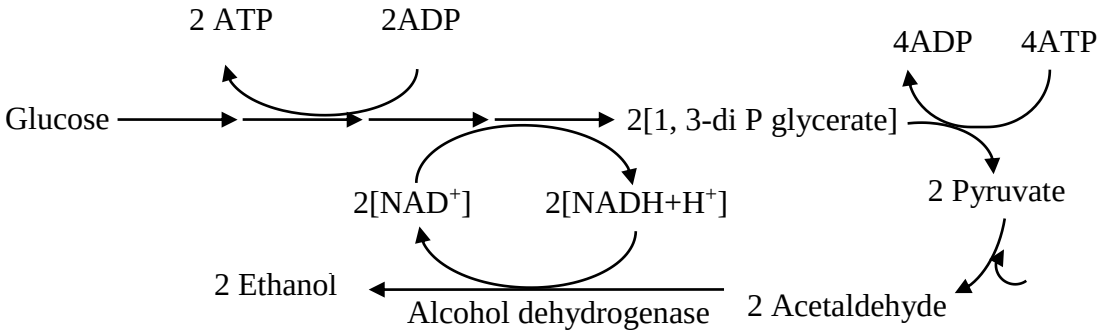

Fig. 2.6 Simplified pathway of alcohol synthesis by yeast

Source: Baxter and Huges, (2001)

2.5.3 Production of toxic compounds

In addition to the production of carbon dioxide and ethanol by fermentation, microorganism also imparts production of toxic compounds mainly methanol, aldehydes, higher alcohols etc. along with some organoleptic compounds which are important for evaluating the flavor of the beverages (Bisson, 2001). Alcoholic fermentation of fruits and grains with yeast usually Saccharomyces cerevisae, yields ethanol and very small amount of other organic compounds. Occasionallymethanol will contaminate the final product which may arise by demethylation of pectin by pecticesterase enzymes. However, it can be carefully removed by distillation (Berglund, 2004).

Yeast doesn't form an enzyme capable of hydrolyzing pectin but pectin esterase is abundant in fungi. According to Pilnik *et al*., (1981) fungi of species *Penicillium, Fusarium, Rhizopus,* and *Seperotina* etc. contain pectin esterase enzymes. Similarly bacteria of species *Clostridium* also contain this enzyme. The ability of enzyme rises as the pH increases from 1 to 6 and the production of methanol goes up. Thus, if the grain with relatively high pH becomes contaminated with mold, the amount of methanol formed may be fairly high (Berglund, 2004).

$$CH_3OH \xrightarrow{\text{Alcohol}} HCHO \longrightarrow HCOOH$$

Fig. 2.5 Production of formaldehyde and formic acid from methanol

Source: Osobamiro, (2013)

Major toxic effects of methanol are caused by formaldehyde and formic acid which are produced when methanol is oxidized by alcohol dehydrogenase in liver and kidney, as presented in Fig. 2.5. The former is responsible for the damage of rational cells that may cause blindness while the latter produces severe acidosis that may eventually lead to death. A minor effect of methanol is depression of CNS (Osobamiro, 2013).

Aldehyde is formed during glycolysis. It is synthesized by yeast as intermediates in the formation of alcohols through the decarboxylation of ketoacids and is released under two conditions; when ethanol formation is blocked due to absence of alcohol dehydrogenase, or when NADH is being used for some other purpose and does not need to be recycled during end product production. Thus, the excess quantities of acetaldehydes are produced when the reduction, catalyzed by alcohol dehydrogenase is rate limiting (Engan, 1981).

The corresponding aldehydes to most of the alcohols formed by yeast have been detected in much alcoholic fermentation. Blood acetaldehyde level above 0.5 mg/ dl is toxic and its exposure limit is 100 ppm (Bisson, 2001).

Among 45 types of alcohols, higher alcohols like n-propanol, isobutanol, amyl alcohol are most important in alcoholic beverages, most of these can be derived from the carbon skeletons of common amino acids by catabolic sequences of reactions shown in Fig. 2.6.

$$NH_2 \longrightarrow RCH_2COOH \longrightarrow RCOCOOH \longrightarrow RCH_2 + CO \longrightarrow RCH_2OH$$

Valine	$\longrightarrow$	Isobutanol
Leucine	$\longrightarrow$	isoamyl alcohol
Isoleucine	$\longrightarrow$	optically active amyl alcohol
Phenylalanine	$\longrightarrow$	2-phenyl alcohol

Fig. 2.6 Production of higher alcohols

Source: Berry and Watson, (1987)

Adding ammonium salts as a nitrogen source can also stimulate higher alcohol formation, Fermentation under pressure (0.8-2 atm) reduces higher alcohol formation and can be used to control the excessive higher alcohol formation, which occurs at higher fermentation rates and obtained at higher temperature. Isopropyl and n-propyl alcohols are twice as toxic as ethanol. The fatal dose by indigestion is 250 ml. About 15% of ingested

dose of isopropyl is metabolized to acetone. The principal manifestation of acute isopropyl alcohol poisoning is CNS. Fatal dose of amyl alcohol is 200 ml.

2.5.4 Production of flavoring compounds

The volatile component profiles of alcoholic beverage products consist of a wide range of compounds, including acids, alcohols, aldehydes, and other trace level flavor compounds (Egan *et al.*, 1984). Flavour compounds spirits originate from the raw materials used for fermentation and from alcoholic fermentation by yeasts (*Saccharomyces cerevisiae*) and other microorganisms which metabolize carbohydrate, amino acids, fatty acids and other organic compounds. Fermentation of carbohydrates not only leads to the main products ethanol, glycerol and carbon dioxide, but also a typical fingerprint of volatile metabolites at relatively low levels, like aldehydes, ketones, higher alcohols, organic acids, and esters, which are called fermentation by-products or 'congeners'. Some of the volatile substances which are produced during fermentation, like acrolein, diacetyl, or acetic acid, are the result of enhanced microbiological activities and may cause an unpleasant flavour (off-flavour) at certain levels; thus, elevated concentration of such compounds are markers for spoilage of the raw material, negative microbiological influences during or after the fermentation processes, or a poor distillation techniques (Berger, 2007).

2.5.4.1 Carbonyl compounds

Acetaldehyde is the major important carbonyl compound of alcoholic fermentation and is formed as an intermediate compound formed by degradation of pyruvate; its production by yeasts depends on the pyruvate decarboxylase activity of the yeast. Since acetaldehyde is one of the most volatile compounds, the highest levels are in the 'head cut' of the distillation and thus can be separated from the 'heart cut'. 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).

2.5.4.2 Esters

An ester is a compound formed from the reaction between a carboxylic acid and an alcohol. 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. Esters are formed by the combination of reactive acids and alcohols. Since the alcohol present in by far the largest quantity is ethanol, most of the esters produced are ethyl esters. Two examples are ethyl acetate (solventy, slightly gluey aroma) and ethyl hexanoate (red apples and aniseed aroma). The flavour balance of the esters produced is dependent on the yeast strain used (Egan *et al.*, 1984).

2.5.4.3 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, cc-ketoglutarate, malate and citrate are derived from pyruvate via limited tricarboxalate acid cycle, Pyruvate itself constitutes a qualitatively important group of acids. They may have direct effect on flavour (e.g. the mouth feel flavour 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 faftyacid synthetase pathway. 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 from the main biosynthetic pathway. These fatty acids are important flavor compounds in their own right and have been reported to give a caprylic, goaty, soapy or fatty flavor to beer and when released by autolysis during the maturation of beer they have been associated with a yeasty flavor (Li and Liu, 2015).

The acids present may be volatile or fixed. The term, volatile acid is rather loose one. It refers to the volatile fatty acid with steam. Besides acetic acid and lactic acid which is the normal by-product of alcoholic fermentation; formic, butyric, propionic and traces of other fatty acids are present. Acetic acid is not only a by-product of alcoholic fermentation but during the course of fermentation an appreciable amount may be utilized by the yeast. The

volatile acids are produced mainly during the initial stage of alcoholic fermentation. More is formed in presence of oxygen than its absence (Mengel *et al*., 2001).

2.5.5 Fermentation of cassava

Fermentation of cassava is an important processing technique followed in different parts of the world. Although fermentation is known to bring about vast changes in the physicochemical and functional properties of the tubers, attempts have seldom been made to consolidate and critically analyze the available information. Glaring inconsistencies and contradictions noticeable in some of the results reflect the differences and variations in the artisanal processes followed in the preparation of these products. It also stresses the need for a systematic study of not only the quoted products, but also a number of other fermented cassava products that have not been well documented (Moorthy and Mathew, 1998)

The fermentation process of staples serves as a means of providing a major source of nourishment for large rural populations, and contributes significantly to food security by increasing the range of raw materials, which can be used in the production of edible products. Fermentation enhances the nutrient content of foods through the biosynthesis of vitamins, essential amino acids and proteins, by improving protein quality and fibre digestibility. It also enhances micronutrient bioavailability and aids in degrading anti-nutritional factors. This method of upgrading the protein content of cassava had been developed in some countries (Oboh and Elusiyan, 2007).

Some of the fermented products of cassava indigenous to various parts of world are briefly described as follws:

2.5.5.1 *Gari*

Gari is a fermented cassava product widely consumed in many West African countries. The cassava tuber is harvested, peeled and washed, grated, and packed into coarsely knit bags. A weight is put on the bag to express some of the juice. It is then left to undergo natural fermentation for several days. The grated cassava, after sieving to remove any coarse lumps and impurities, is heated by means of constant turning over a heated steel pan. On garifying, the grated cassava is dried to about 10% moisture content and the starch

is probably partially dextrinized. At this stage a little palm oil may be added to give it color; the final dry granular product is *gari* (Oyewole and Odunfa, 1988).

2.5.5.2 *Fufu*

Fufu is the meal of soaked fermented cassava and is popular in African countries. The tubers are peeled, washed, cut into thick chunks (20 cm long), and soaked in water contained in earthen-ware pots or in a slow flowing stream for 4 to 5 d. During this period, the cassava tuber ferments and softens, releasing HCN into the soak water. A characteristic flavor of retted cassava meal is also produced. The retted tubers are disintegrated in clean water, sieved, and the starchy particles that go through the sieve are allowed to settle for about 3 to 4 h. The water is decanted while the sediment is packed into a cloth bag, tied, squeezed, and subjected to heavy pressure to expel excess water. The resulting meal is rolled into balls, and cooked in boiling water for about 30 to 40 min. The cooked mass is pounded in a mortar with a pestle to produce a paste, *fufu*, which can be eaten with sauce, soups, or stew (Moorthy and Mathew, 1998)

2.5.5.3 *Lafun*

Lafun is a fibrous powdery form of cassava similar to *fufu* in Nigeria. The method of production of *lafun* is different from that of *fufu*. In the traditional preparation, fresh cassava roots are cut into chunks and steeped for 3-4 days or until the roots become soft 'Lafun' is a fine, powdery product which is prepared by the fermentation of cassava (Manihot esculenta Crantz) tubers. It is different from other cassava products like 'gari', 'fufu', 'achicha', 'akpu', 'pukuru', etc. It is usually made into a porridge in boiling water before consumption. Unlike other cassava products, the consumption of'lafun' is restricted to the western parts of Nigeria where it is common with the Yorubas of Ogun and Oyo States. This restricted area of consumption could have been responsible for the few investigations into this food (Oyewole and Odunfa, 1988).

2.5.5.4 *Chikwangue*

Chickwangue is the most popular processed food form of cassava in Zaire. ‘Myondo’ and ‘Bobolo’ in Cameroon, ‘Mboung’ in Gabon, ‘Mangbele’ in Central African Republic belong to this group. Similar products are consumed in Congo, Sudan, and Angola (Moorthy and Mathew, 1998)

2.5.6 Effects of fermentation on cassava

Microbial fermentation has played a significant role in the nutritional enhancement of erstwhile worthless and often discarded agro-industrial by-products generated through the harvesting and processing of cassava roots. Myriads of laboratory analysis and animal trials have revealed the nutritive values of microbially fermented cassava products and by-products. Fermentation has also helped in mitigating against the level of anti-nutrients in these products while at the same time helping to beef up the relatively low calorie content of such by-products like cassava peels (Aro, 2008).

In view of the low protein content, lack of essential amino acids and high cyanide content of some cassava products, methods of upgrading the protein content of cassava and reducing the anti-nutrient content (especially cyanide) are being developed. Solid media fermentation of cassava products with certain micro-fungi has the advantage of being able to increase the nutrient and reduce the anti-nutrient content. In a study conducted by Oboh and Elusiyan (2007) in order to observe the ability of pure strain of *R. oryzae* and *S. cerevisae*, to increase the nutrient and decrease the anti-nutrient of cassava flour, low and medium cyanide variety of cassava were used. In their study they reported that the significant amount of increase in protein, ash and fat can be observed during the fermentation process. In the same study they observed that carbohydrate and crude fibre content of cassava decreased significantly. The basis for the decrease in the crude fibre and carbohydrate content of cassava flour can be understood as the action of hydrolytic enzymes secreted by micro fungi. These enzymes are capable of hydrolyzing both the carbohydrate and crude fibre into simple sugars, which the organism could use as its carbon source and transform it to other macromolecules or metabolites such as protein, fat, alcohol etc. (Oboh and Akindahunsi, 2003). Also their report showed the significant decrease of cyanide content of cassava flour upto 3.4 mg/kg for low cyanide variety of cassava and upto 8.5 mg/kg for medium cyanide variety. Their study suggested that *R. oryzae* and *S. cerevisae* are capable of utilizing cyanogenic glycosides and the breakdown products, thus explaining why they are suitable natural flora involved in cassava fermentation (Akindahunsi *et al.*, 1999).

Part III

Materials and methods

3.1 Materials

3.1.1 Cassava

Cassava was bought from the local fruits and vegetable market of Dharan. Cassava tubers were then cleaned and sorted to remove dust, foreign matters and damaged parts. Clean and sound tubers were kept in a separate container and were selected for further processing. Basic required analysis of raw material was performed in raw form. Tubers were then cooked by boiling before they were subjected to fermentation process. Since the cassava tubers were locally produced under no technical supervision, it was hard to find the variety of available cassava. Also Shrestha (1992) has mentioned that two semi sweet varieties of Cassava were introduced in Nepal, namely 'Cylone' and 'Mysore'. Since then no researches or written documents are available regarding the variety of cassava cultivated in Nepal. So the the cassava here used are assumed to be one ofe those two varieties.

3.1.2 *Murcha*

Murcha required for the inoculation of fermentation culture were obtained from the local market of Dharan.

3.1.3 Plastic jars

Plastic jars purchased from local department store of Dharan were used to carry out overall fermentation process.

3.2 Methods

3.2.1 Preparation of cassava *jand*

In order to prepare cassava *jand*, completely traditional method was used. Method for preparation of *jand* cassava collected from local market of Dharan were cleaned and peeled first. Required chemical analysis of raw cassava roots was done then the boiling of cassava tubers were done for about 30-40 min until they were soft. Thus cooked cassava tubers

were allowed to cool till just warm. It was then mashed up slightly to make comparatively smaller chunks. Inoculum of *murcha* was then sprinkled all over the cassava uniformly and mixed thoroughly. Then the inoculated mass was transferred in 10 plastic jars of about 500 ml capacity. Jars were covered with a small piece of muslin cloth for initial one and half days and kept in warm place in order to ensure the proper microbial biomass build up. After then they were closed tightly with lids to make anaerobic condition. Every jar was subjected to same environmental conditions. One jar at a time was used as a sample to analyse the progress of fermentation in every another 3rd day. Use of separate jars minimized the risk of contamination while drawing the sample every time. Analysis of sample was carried out upto 21st day of fermentation. Method of making cassava *jand* applied here is inspired from the traditional method described by (Kharel *et al.*, 2009) with slight modifications as suggested by local people makers of cassava *jand*. Method of preparation of cassava *jand* followed in this experiment is shown in Fig. 3.1.

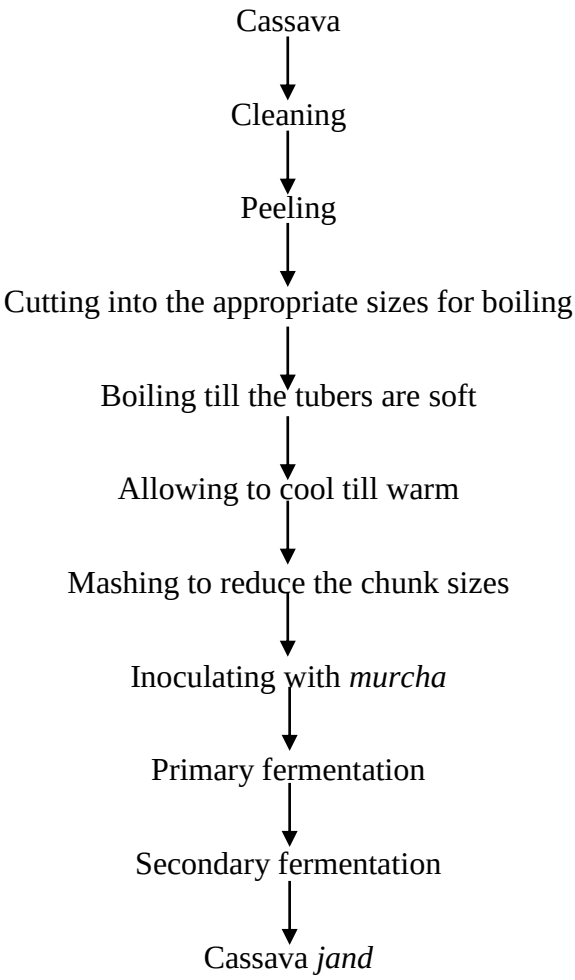

Fig. **3.1** Preparation of cassava *jand*

All the processes here were conducted in ambient room temperature in order to replicate the authentic traditional method of cassava *jand* preparation.

3.2.2 Analytical methods

3.2.2.1 Preparation of sample for analysis

Cassava tubers collected were thoroughly cleaned and peeled. In order to prepare *jand* as per the traditional method, process described by Kharel *et al.* (2009) was followed with slight modifications as suggested by the local cassava *jand* makers. Samples were fermented in small plastic jars of about 500 ml volume. All the jars were subjected to same fermentation conditions and one jar at a time was used to draw the sample for various analyses during the various stages of fermentation. This was done in order to prevent the microbial contamination in the product during the process of withdrawing samples.

3.2.2.2. Determination of pH and TSS

pH was determined by pH meter (Hanna Instrument) and TSS by portable refractometer (Hanna Instrument).

3.2.2.3 Determination of total reducing sugar

Total reducing sugar was determined by Lane and Eynon method as described by Ranganna (1986). 25 g of neutralized sample was clarified and maintained with distilled water in 250 ml volumetric flask. 10 ml of such sample was titrated with standard Fehling mix solution, and calculation of total reducing sugar was carried out as % dextrose.

3.2.2.4 Determination of total, fixed and volatile acidity

Total, fixed and volatile acidities were determined as per Ranganna (1986). 10 ml of sample was titrated with 0.1 N NaOH using a few drops of 1% phenolphthalein solution as indicator and calculation of total acidity was done as per cent Lactic acid. The sample was boiled for 20 min, maintaining the volume with distilled water, and titrated with same manner for fixed acidity as % lactic acid. The difference between both titer was taken and calculated for volatile acidity as % acetic acid.

3.2.2.5 Determination of alcohol content

Alcohol by volume was determined by pycnometric method as per official method 935.21 described in AOAC (2005). 50 ml of each sample and 50 ml of distilled water was added to it; was distilled till 40 ml of distillate and volume maintained to 50 ml by distilled water. Then weight of dry picnometer, distillate in piconmeter and water in picnometer was taken; and room temperature was noted. The specific gravity of water was calculated, and alcohol % (v/v) was found by chart.

3.2.2.6 Determination of protein content

Crude protein content of cassava and cassava *jand* samples were determined as described by AOAC (2005)

3.2.2.7 Determination of ester content

Total ester content was determined as per Kirk and Sawyer (1991) with minor modifications. Briefly 50 ml of beer distillate was taken in a reflux flask and neutralised using 0.1 M NaOH and 1% phenolphthalein indicator. After this % ml of 0.1 M NaOH was added and condenser was connected. It was then refluxed for 1h, cooled and titrated with 0.5 M H_2SO_4. Finally easter content was calculated using following expression:

$$\text{Ester content } (\text{g ethyl acetate/ L alcohol}) = \frac{880 \times \text{ titre}}{\text{\% alcohol by volume}}$$

3.2.2.8 Determination of total aldehyde content

Total aldehyde as g acetaldehyde/ 100 L alcohol was determined as per Kirk and Sawyer (1991) with slight modifications. Solution A, B, C and D was prepared as per followings:

A: Mix 15g potassium metabisulphite with 70 ml conc. HCI and dilute to 1 L with distilled water

B: Dissolve 188 g $Na_2HPO_4.12H_2O$ + 21 g NaOH and 4.5 g EDTA in water and dilute to 1 L with distilled water.

C: Dilute 250 ml conc. HCI to 1:1 with distilled water.

D: Mix 100g boric acid with 170 g NaOH add water to dissolve and dilute to 1 L with distilled water.

In 1000 ml conical flask 300 ml boiled and cooled water and 10 ml of solution A were taken. To this mixture 40 ml of beer distillate was added, stopper was placed and the flask was swirled. It was then allowed to stand for 15 min. After this, 10 ml of solution B was added, mixed by swirling and allowed to stand for further 5 min. Then 10 ml of solution C and 10 ml fresh 0.2% starch solution was added, mixed by swirling and iodine was (can 0.1 M) added so that excess bisulphate was just destroyed and colour of the solution became faint blue. Finally 10 ml of solution D was added and the liberated bisulpite was titrated with 0.05 M iodine solution to the same faint blue end point. Total aldehyde as g acetaldehyde per 100 L alcohol was calculated using following expression:

$$\text{Total acetaldehyde (g acetaldehyde/ 100 L alcohol)} = \frac{\text{Titre} \times 2.2}{S}$$

Where, S = % alcohol by volume in the sample

3.2.2.9 Determination of cyanide content

Residual Cyanide or HCN content in was determined by the method conducted in the Technology Center for Agribusiness Analysis at the Catholic University (CeTeAgro / UCDB) in Campo Grande, State of Mato Grosso do Sul, Brazil. This method is an improvement of the method of (Baltha and Cereda, 2006). (Baltha and Cereda, 2006) have used a simple method for determining the free and potential cyanide based on autolysis of Scassva roots (Bradbury *et al.*, 1991) in which endogenous linamarase enzyme was used to hydrolyze linannarin and release CN. Free cyanide was determinate by adapting the method of Smith described by, which is based on colorimetric reaction of picrate solution (Brito *et al.*).

Here the initial analysis of raw and boiled cassava, cassava tubers, raw and boiled were grinded. Cyanogenic glycosides were allowed to hydrolyse to hydrocyanic acid. Sulfuric acid was used to stop the reaction and increase the stability of reading and finally HCN content was determined colorimetrically (SSJ-1104 spectrophotometer). Similar process was repeated with the fermented mash of cassava but relatively less grinding or simple mashing was enough for fermented semisolid mass.

3.2.2.9.1 Reagents required

- Picric acid solution: Saturated solution of picric acid was obtained by diluting 2.56 gram in 100 ml of distilled water.
- 5% sodium carbonate solution (Na_2CO_3): 5 g of sodium carbonate was diluted in 100 ml of distilled water.
- 0.01 M sulfuric acid solution (H_2SO_4): 0.5330 ml of concentrated sulfuric acid was diluted in 1000 ml of distilled water.
- Alkaline picrate solution: Equal volumes of 5% sodium carbonate and picric acid were mixed for having alkaline picrate just in time for analysis.

3.2.2.9.2 Procedure followed

5 g of weighed and prepared sample was taken in clean mortar and pestle. Then extraction was carried out in 50 ml distilled water by grinding it homogenously for 1 to 2 min. The extract was filtered through a moderately retentive filter paper Whatman No. 42. The filtrate was taken and 5 ml of this aliquot was transferred to a clean test tube followed by the addition of 10 ml of alkaline picrate and 5 ml of distilled water. This was sample solution. Similarly, blank was made with each set of samples containing 0 ml of sample filtrate, 10 ml of alkaline picrate and 5 ml of distilled water. The test tubes containing sample and color reagents were incubated for 15 min in water bath at 37°C. Then before reading were added 15 µl of sulfuric acid to stop the reaction and increase the stability of reading. All the sample and blank was shaken well and the absorbance was immediately read at 535 nm (Brito *et al.*, 2009).

3.2.2.9.3 Preparation of standard curve and calculation

Standard curve couldn't be plotted in the laboratory due to lack of chemical KCN or HCN. Thus, standard curve made and plotted in the Technology Center for Agribusiness Analysis at the Catholic University (CeTeAgro / UCDB) in Campo Grande, State ofMato Grosso do Sul, Brazil was used for our study also. In this experiment the curve pattern was Y = 0.276X + 0 009 (R_2 = 0.9536) and free cyanide is expressed in milligrams (mg) by substituting X for absorbance obtained from the sample solution reading. The equation this best fit curve was then used in calculating HCN content (unknown parameter) by substituting the known parameter absorbance as X for each sample. From this equation Y

was calculated in mg/ml. Finally the HCN content in sample solution was obtained by using following formula.

HCN content (ppm or mg/kg) (Y* 50* 1000)/ (aliquot taken*sample weight taken)

3.3 Statistical analysis

For all chemical analysis triplicates of the same sample were used for the determination of each constituent. Mean values with standard deviation were computed. Data were subjected to analysis of variance and read at 0.95 confidence level using statistical software GenStat Release 7.1 (Discovery Edition 3 developed by VSN International Limited).

Part IV

Results and discussion

Cassava was bought from the local market of Dharan. Proximate analysis of raw material was performed. Cassava tubers were then peeled cut into suitable chunks and were boiled for 30-45 mins until they were thoroughly cooked and were soft in texture. Cassava *jand* was prepared in the laboratory following the traditional methods used in the common household of various *jand* consuming ethnic communities of Nepal. *Jand* was inoculated with locally available *murcha* and allowed to ferment in small plastic jars. Every jar was subjected to same environmental conditions in order to ensure the uniformity in fermentation process. One jar at a time was withdrawn as a sample which prevented the contamination and thus spoilage of the product. Samples were withdrawn in the interval of every three days and analysed for alcohol, pH, protein content, cyanogens content, TSS and reducing sugars.

4.1 Proximate analysis of raw cassava

After the cassava tubers were cleaned and peeled, proximate analysis of raw cassava tubers was carried out. Proximate composition of raw cassava tubers as obtained in the laboratory are tabulated in the Table 4.1.

Table 4.1. Proximate composition of raw cassava tubers (wet basis)

Parameters	Composition (%)
Moisture	53.17 (0.63)
Fat	0.3 (0.75)
Protein	1.84 (0.21)
Crude fiber	2.1 (0.18)
Ash	1.03 (0.5)
Carbohydrate (by difference)	41.56

*Values are the means of three determinations. Figures in the parenthesis are standard deviations.

The proximate composition of raw cassava obtained in the laboratory is somehow comparable to the data provided by USDA (2016). Slight differences might have occurred in various parameters due to the differences in analytical methods or due to the difference in variety of cassava used.

4.2 Changes in alcohol content

As the fermentation progressed, the alcohol content of the brew increased to 2.43±0.1212, 5.47±0.155, 9.32±0.4325, 12.403±0.4186, 13.183±0.1617, 13.697±0.1457 and 13.637±0.0874 in day 3, 6, 9, 12, 15, 18 and 21 respectively. The graphical representation showed almost steep rise in alcohol content of brew upto 12th day of fermentation. After that the rate of alcohol formation seems to have become slow and finally stopped during final 18th and 21st day of fermentation. Statistical analysis showed that the fermentation time had significant effect on the alcohol content of cassava *jand*. Statistical analysis carried out at $p<0.05$ showed that the alcohol content of cassava increased significantly during all other days of fermentation gradually except final 18th and 25th days of fermentation where no significant difference in the alcohol content was observed. The change in alcohol content of cassava *jand* during various intervals of fermentation is shown in Fig. 4.1.

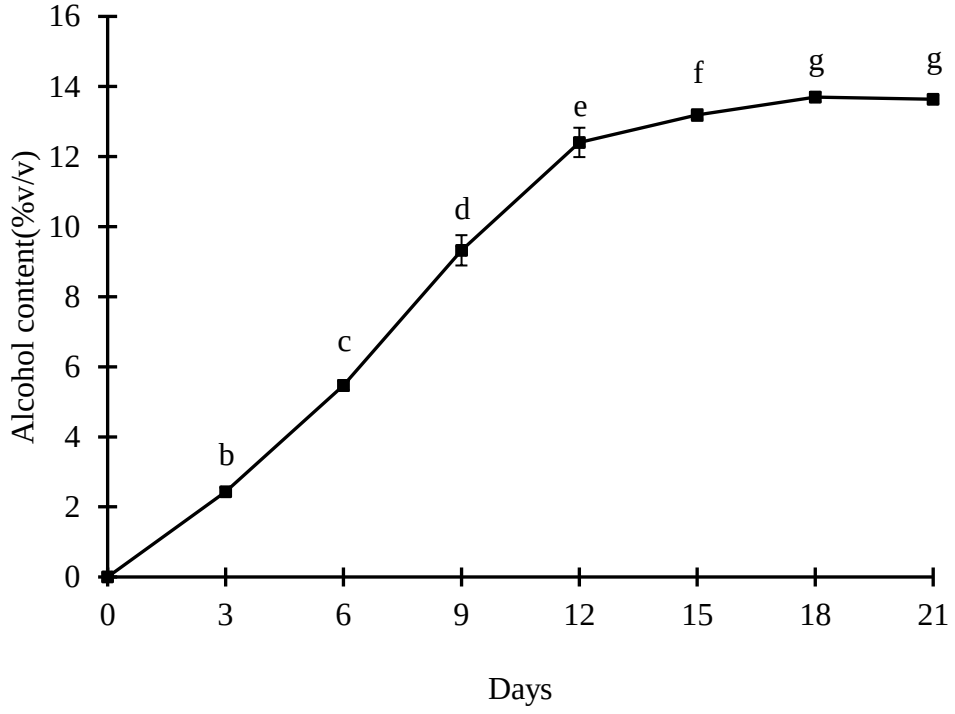

Fig. 4.1 Changes in alcohol of cassava *jand* fermentation process

Due to the lack of adequate researches on *jand* prepared from cassava, appropriate comparison couldn't be made but during the preparation of *jand* from other starchy substrate i.e. rice, Karki and Kharel (2011) reported the alcohol content of brew of finger millet *jand* to be 15.81% during the fermentation period of 15 days. Though the starter culture used in these works were somehow similar, the differences in reading may be due to the slight differences in micro flora and differences in fermentation time and temperatures. Also the change in raw material which also altered the amount of fermentable sugars available also might have affected the final alcohol content of the product.

4.3 Changes in reducing sugars

Initially, there was no detectable amount of reducing sugars in the unfermented cassava as shown by Lane and Eynon method. As the fermentation progressed, the reducing sugar content of the brew rapidly increased to 1.71±0.462 and 11.87±0.136 in day 3 and 6 respectively, then again decreased to 6.033±0.065 and 0.567±0.060 in next 9^{th} and 12^{th} days of fermentation.

Very low amount of reducing sugars were assumed to be present during the initial phase prior to the fermentation. Later a sharp rise in reducing sugar was observed upto 6^{th} day of fermentation and again steep decline in reducing sugars was noticed and finally was again difficult to detect after 15^{th} day offermentation.The initial increase in the amount of reducing sugar content of the fermenting mash may be due to the hydrolyzing action of molds on the starch content of the tubers. Later, the decrease of sugar can be due to the conversion of available sugars in the media to alcohol by the yeasts. Statistical analysis of data at $p<0.05$ showed significant changes in reducing sugars of cassavain 3^{rd}, 6^{th}, 9^{th} and 12^{th} day of fermentation. During 1^{st}, 15^{th}, 18^{th} and 21^{st} day of fermentation, values are shown to be 0 for reducing sugars which shows that the amount of reducing sugar was so low that it couldn't be detected by the method followed.

Variation observed in the amount of reducing sugars of fermenting *jand* is graphically represented in Fig. 4.2.

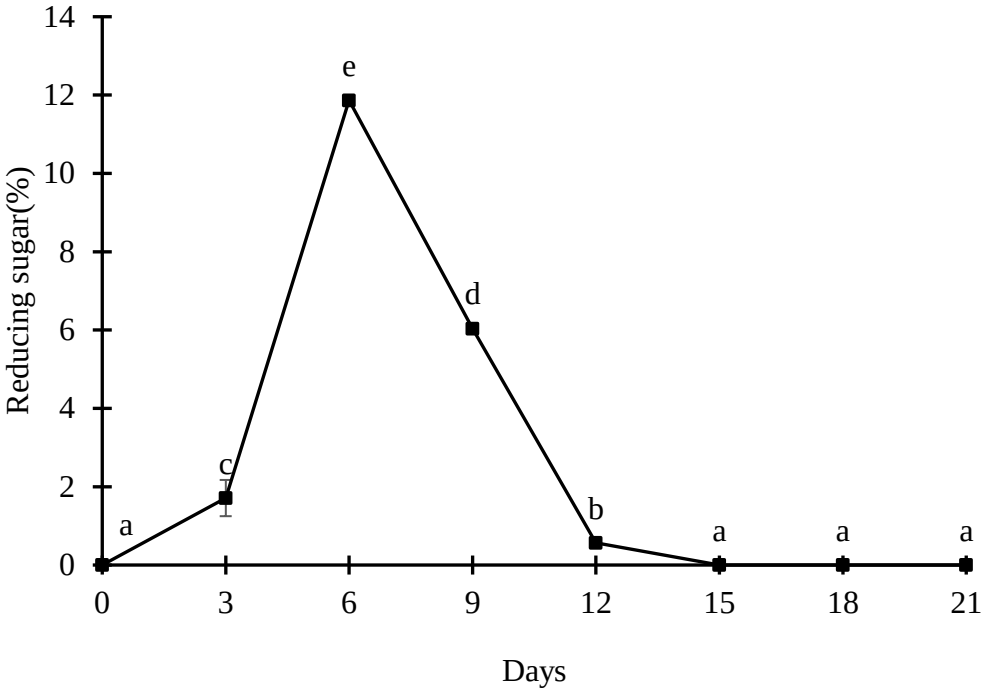

Fig. 4.2 Changes in reducing sugar during different stages of fermentation

Dangol (2014) also found similar results in his work where the reducing sugar content of fermentation broth increased rapidly upto 6th day of fermentation and again decreased after that.

4.4 Changes in TSS

In this case of fermentation, as the fermentation progressed, the TSS of the brew increased to 10.5±0.1ºBx, 19.03±0.21ºBx, in day 3 and 6 respectively and gradually decreased to 16.467±0.057ºBx, 11.33±0.057ºBx, 8.17±0.57ºBx in day 9, 12 and 15 and it was recorded to be constant 7 in further 18 and 21 days of fermentation. Initially no liquified sample was obtained as the fermentation wqas carried out on boiled solid chunks of cassava tubers. On 3rd day of fermentation small amount of semi solid tyoe of mass was observed an dfound the TSS to be 10.5±0.1ºBx. Reading of 6th day showed the highest value of TSS which later decreased gradually with the progression of fermentation. After 12th day of fermentation, decreasing pattern of TSS slowed down significantly and came to almost a halt during final days of fermantation. Statistical analysis at $p<0.05$ showed no significant differences in the TSS of *jand* in the final 18th and 21st days of fermentation.

Increase in TSS might be due to the saccharification of starch by the enzymes elaborated by the mold and then decrease in TSS might be due to the utilization of sugar by the yeasts for the alcohol fermentation. Change in TSS during different stages of fermentation is shown in Fig. 4.3.

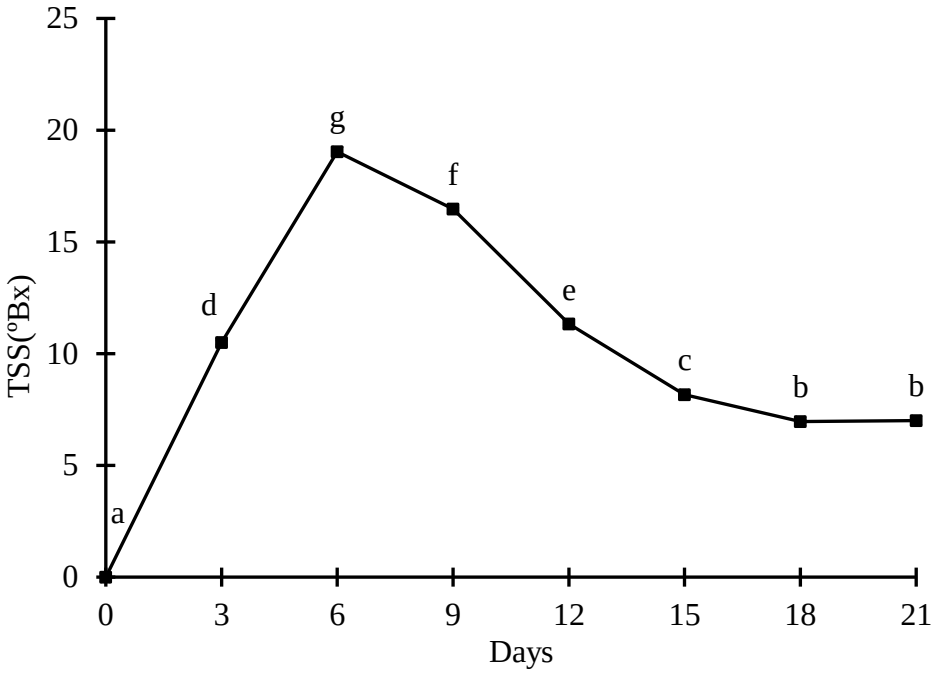

Fig. 4.3 Changes in TSS of cassava *jand* in different stages of fermentation

Karki and Kharel (2010) found that TSS of mass during the semi-solid fermentation of finger millet went decending upto 3.27ºBx, while Dangol (2006) also reported the final TSS of broth to be 7ºBx in *hyau thon.*

Primarily, differences in fermentation conditions like temperatures, fermentation vessels and source of starter cultures may be the root cause behind the differences observed. Besides this *murcha* might vary widely depending on the process followed to prepare them and source of flora used.

4.5 Changes in pH

When the entire process of fermentation was observed, as the fermentation progressed, the pH of the mash was initially recorded to be 6.11±0.095. this further decreased to 4.88±0.035, 4.12±0.025, 3.81±0.021, 3.803±0.021, 3.79±0.01 in the day 3, 6, 9, 12 and 15

days respectively. During the final 18 and 21 days of fermentation pH of the mash was recorded to be almost constant i.e. 3.65±0.015 and 3.64±0.01 respectively.

Since the *jand* being traditional beverage no standardization of pH was done before the inoculation of starter. Initial pH of 6.11±0.095 slowly decreased and was observed to be almost constant after 9th day of fermentation. A significant change was observed in the pH of fermenting cassava in all days except final 18th and 21st days of fermentation. Change in pH of fermenting brew is shown in Fig. 4.4.

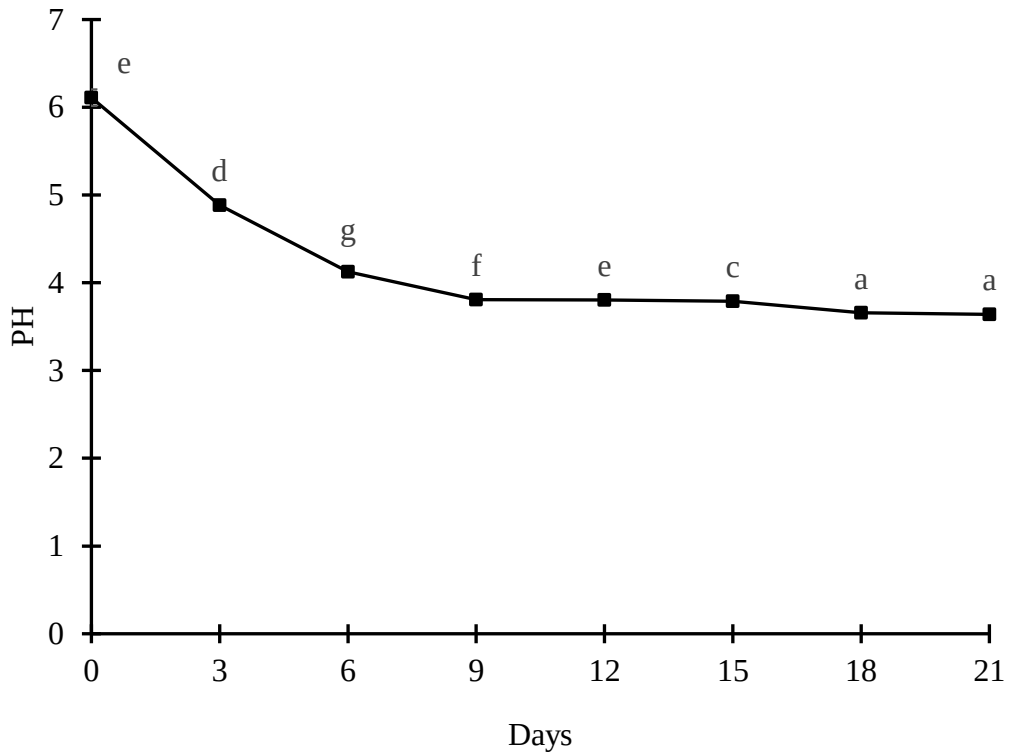

Fig. 4.4 Changes in pH of cassava *jand* during different stages of fermentation

Yeast grows well and works effectively at a pH of 4.5 to 5.5 for alcoholic fermentation .The souring procedure in the initial stage has a great role in maintaining the pH of the mash. In addition to bringing about desirable aesthetic quality, it helps to protect the mash from undesirable bacterial contamination.

According to Karki and Kharel (2010) pH of fingermillet *jand* went upto 3.37. also in another work, Karki and Kharel (2011) reported the pH of *jand* made from different cereals to be ranging from 4.17 to 3.9. Hence, we can conclude that the readings of pH observed here are comparable to various other researches conducted elsewhere.

4.6 Changes in cyanogens

Cyanogenic glycosides in terms of mg of free HCN liberated per kg sample in raw cassava was found to be 75.124±0.43 mg/kg fresh wt. Since boiling helps to reduce the cyanogens content significantly, the cyanogens content in boiled cassava was calculated to be 45.17±0.1713 mg/kg. Considering this the initial cyanogens content in the mash to be fermented, the gradual decrease in the content of cyanogens was observed. Cyanogen content in the brew was found to be 41.246±0.7608 mg/kg, 31.388±0.3725 mg/kg, 23.702±0.4121mg/kg and 16.091±0.3871mg/kg in day 3, 6, 9 and 12 respectively. After that, somewhat almost constant values i.e. 7.5217±0.4498 mg/kg, 7.1557±0.2011 mg/kg and 6.911± 0.0992 mg/kg were observed in final 15, 18 and 21 days of fermentation. At $p<0.05$, there was significant change in the cyanogens content of fermenting cassava mash upto 15th day of fermentation, whereafter no significant change was observed.

Cyanogenic glycoside detected in terms of free cyanide was, found to decrease sharply and significantly upto 15th day of fermentation. Statistical analysis showed no significant decrease in cyanogens after that. Decrease in cyanogenic glycosides is graphically represented in Fig. 4.5.

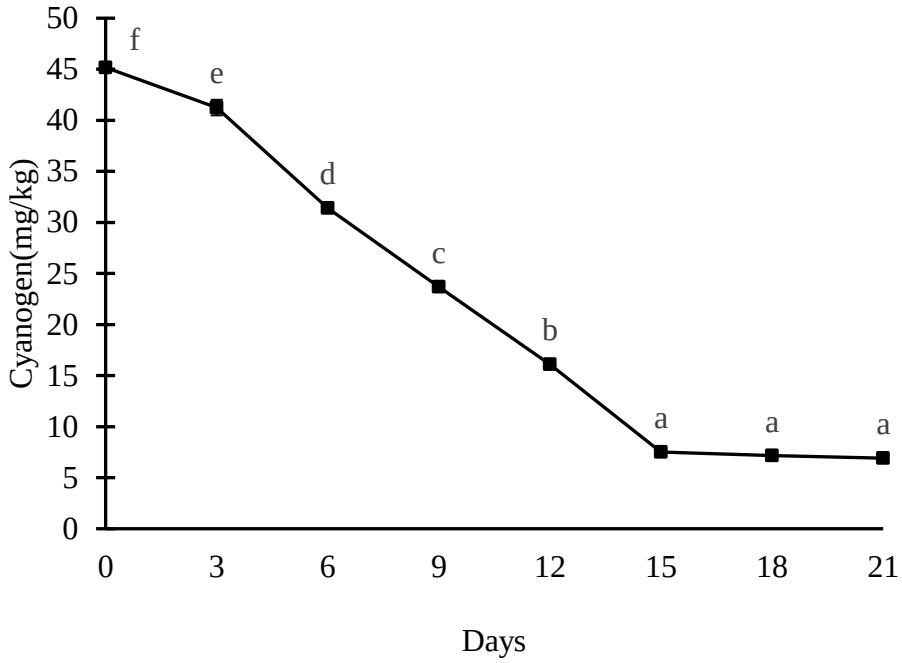

Fig. 4.5 Changes in cyanogens content of cassava during different points of fermentation

Cyanogenic glucosides content of cassava tubers may vary from 15 mg HCN/kg fresh wt. upto 400 mg HCN/kg fresh wt depending on the varietiy of cassava, bitter varieties

containing relatively higher amount of cyanogens (Padmaja and Steinkraus, 1995). In the same research Padmaja and Steinkraus (1995) mentioned that there is significant loss of cianogens upto 27-47% during boiling. Also, FAO (1991) has marked 10mg/kg dry wt as the safe limit for cyanogen content of anyy food products.

Cassava used in this work being the one of the sweet varieties, the initial cyanogen content of the raw cassava was found to be in the described range. Also the decrease in the cyanogens content after boiling is found to be of nearly 40% as described in previously mentioned works. The final cyanogens content of cassava *jand* here prepared is quite below thw FAO standards and hence we can say that the traditional Nepalese alcoholic fermentation process is capable of rendering the final product safe from the possible toxicity caused by cyanogenic glucosides in cassava. Though unknowingly, the process of making *jand* from cassava is making this toxic food more safer for consumption than any other modes followed in context of Nepal.

4.7 Changes in protein

Cassava is mainly a starchy tuber and fails to meet the requirement of protein rich food. The initial percentage of protein in raw cassava tuber was found to be 1.8367±0.0681. Though it may not be nutritionally significant, the gradual increase in available protein content i.e. 1.98±0.02, 2.881±0.029, 2.955±0.0564 in day 3, 6 and 9 respectively upto 3.195±0.168 in day 21. At $p<0.05$, no significant increase in protein content of cassava was seen upto 6^{th} day of fermentation. A sharp increase was seen in between 6^{th} and 9^{th} day. A slight increase in the amount of protein was observed in the days thereafter.

Cassava is not a protein rich diet however,that small amount of protein was found to be increased rapidly during 3^{rd} to 6^{th} days of fermentation. Though the amount is small quantitatively, there was a sharp increase of 73.95% available protein content in the fermenting cassava *jand.* Prolifiration of biomass might have helped to increase the amount of available protein in terms of single cell protein. Changes in the amount of protein is shown in Fig. 4.6.

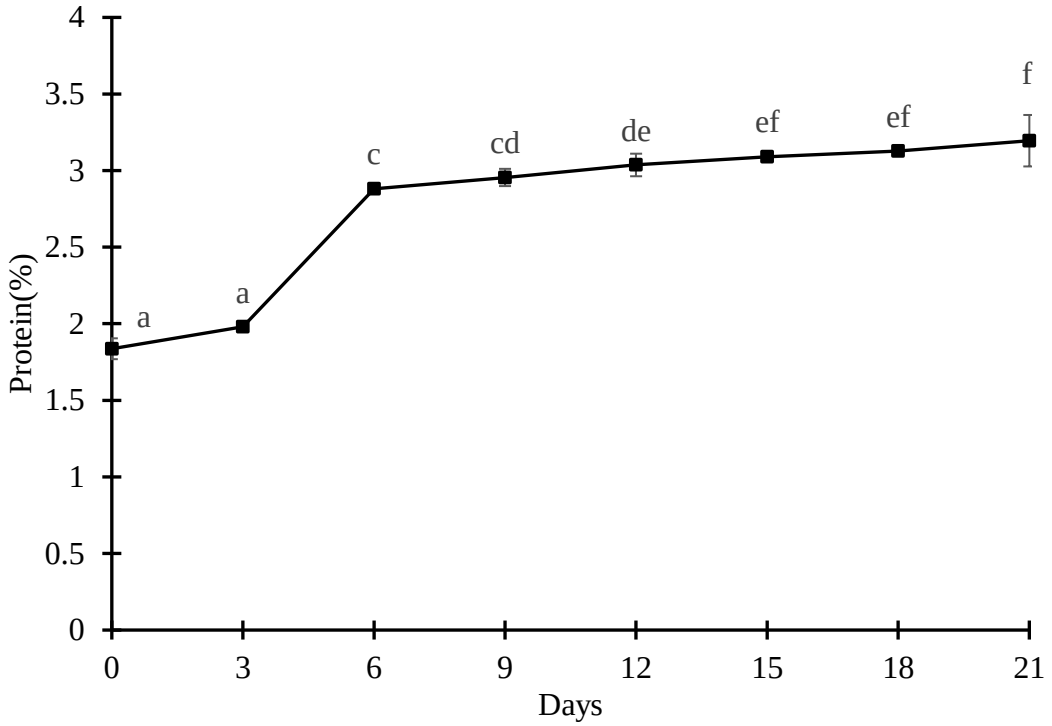

Fig. 4.6 Change in protein during various stages of cassava fermentation

Though cassava doesn't contributes much in as a protein source in diet, this apparent increase in the protein content may be due to the prolifiration of biomass in the fermenting mash, which then increased the amount of available protein as assumed by (Boonnop *et al.* (2009)).

However, no addition of additional mass has been done or occurred during the process in order to justify the increase in protein content. This apparent increase in protein can be justified by the fact that, during ferementation process various volatile components are formed which gradually might have vaporised from the fermenting mass. Also, amount of water and to some extent the alcohol produced might have also been vapouriesd. During fermentation process, biologically active biomass might have also contributed in the loss of overall mass in the form of carbon in carbon dioxide in the course of respiration. All of these losses in mass might have contributed for the apparent increase in the amount of protein per unit mass.

Different chemical changes during the coarse of fermentation is summarised in the Table 4.2.

Table 4.2 Chemical changes during fermentation of cassava *jand*

Parameters	Days							
	0	3	6	9	12	15	18	21
Alcohol (%v/v)	0^{a} (0)	2.43^{b} (0.12)	5.46 ^{c}(0.16)	9.32^{d} (0.43)	12^{e} (0.42)	13.2^{f} (0.16)	13.7^{g} (0.15)	13.64^{g} (0.09)
Reducing sugar (%)	0^{a} (0)	1.71^{c} (0.46)	11.87^{e} (0.14)	6.03^{d} (0.06)	0.56^{b} (0.06)	0^{a} (0)	0^{a} (0)	0^{a} (0)
TSS (ºBx)	0^{a} (1)	19.03^{d} (0.1)	16.67^{g} (0.21)	11.33^{f} (0.06)	8.17^{e} (0.06)	6.96^{c} (0.06)	7^{b} (0.06)	7^{b} (0)
pH	6.11^{e} (0.09)	4.9^{d} (0.03)	4.12^{c} (0.0.2)	3.8^{b} (0.02)	3.8^{b} (0.02)	3.8^{b} (0.01)	3.66^{a} (0.02)	3.64^{a} (0.01)
Cyanogens (mg/kg)	45.12^{f} (0.17)	41.2^{e} (0.76)	31.4^{d} (0.37)	23.7^{c} (0.41)	16.1^{b} (0.39)	7.52^{a} (0.45)	7.16^{a} (0.2)	6.91^{a} (0.1)
Protein (%)	1.84^{a} (0.07)	1.9^{b} (0.02)	2.9^{c} (0.03)	2.95^{cd} (0.05)	3.04^{de} (0.07)	3.1^{ef} (0.02)	3.13^{ef} (0.003)	3.19^{f} (0.17)

4.8 Final aldehyde content, ester content and acidities of cassava *jand*

Cassava *jand* here prepared, had final alcohol content of 13.64 %, final TSS of 7ºBx, Final pH 3.64, protein content 3.19 % and cyanogens content 6.91 mg/kg. Besides these parameters various other chemical attributes that define the quality of of *jand* were analysed after the final product was prepared. Chemical components like aldehydes, esters, acidity are the primary factors which contribute in sensorial quality of *jand* and hence determine the acceptance of product.

Total aldehyde content of cassava *jand* was found to be 6.472±0.49 g/100 L alcohol. During the 15 day fermentation of finger millet *jand* in similar plastic container, Karki and Kharel (2011) reported the aldehyde content of the product to be 0.20 g acetaldehyde/ L alcohol. Lower aldehyde content in cassava *jand* may be the result of different raw material or difference in the quality of micro flora present in the *murcha*.

Total ester content of cassava *jand* was calculated to be 6.9±0.62 g/ 100 L alcohol. Karki and Kharel (2011) reported their readings for same in finger millet *jand* to be 0.787 g/ L alcohol. Here also the difference in raw material, fermentation conditions and micro flora of *murcha* might have responsible for the differences in data.

Total,fixed and volatile acidities of cassava *jand* were calculated to be 1.18±0.04 as % lactic acid, 0.9±0.09 % as lactic acid and 0.147±0.03% as acetic acid respectively. Karki and Kharel (2011) found similar values i.e. 1.05% as lactic acid, 0.94% as lactic acid and 0.075% as acetic acid for total, fixed and volatile acidities respectively.

Part V

Conclusion and recommendation

5.1 Conclusions

In this study cassava *jand* was prepared following the traditional method of fermentation using *murcha* as starter culture and various parameters were analysed in the lab. On the basis of results following conclusions were drawn.

1. Making cassava *jand* is one of the best way followed in Nepal as it not only preserved the ethical values of some communities but also helped inn making the food more safe and nurtitious.
2. As cassava is mainly composed of starch, the changes in various parameters like pH, TSS, reducing sugars and alcohol content are very much comparable to the *jand* made from any other cereals.

5.2 Recommendations

1. Various other processing techniques for cassava available in Nepal and their effect on various nutritional, antinutritional and toxic factors present on cassava can be studied.
2. Raksi can ben made from the distillation of cassava *jand* and its quality and acceptance can be compared with those made from other raw materials.
3. Knowing that this process can make cassava safer and more nutritious, this method can be endorsed as one of the best method of cassava processing and with further researeches done it can be industrialized as well.

Part VI

Summary

Traditional foods, both fermented and non-fermented, have been the basis and are equally important for food security, preservation and cultural and ethical practices.Among various indigenous fermented foods, *jand* and *raksi* are the major alcoholic fermented liquor in various parts of Nepal depending on the availability of the raw materials.

Cassava is one of most important crop that plays the role of energy source for large number of people worldwide in the form of staple diet. Cassava can be cultivated in those places where adequate production of other food crops cannot be expected to meet the daily energy demand of the population. *Jand* is primarily made from cereals, among which finger millet is taken as the raw material of choice for the production of *jand* of better quality. Other raw materials used in *jand* production are rice, maize, wheat and starchy tubers like cassava.

Cassava *jand* is a traditional fermented alcoholic beverage prepared and consumed by *Rai* and *Limbu* communities, especially in districts of eastern Nepal like Khotang, Udayapur, Dhankuta, Sunsari, Jhapa and Ilam. Among the communities cassava *jand* is one of the socially accepted alcoholic beverages. It is turbid thick, cloudy in appearance.

Cassava *jand* was prepared in lab by fermenting the cassava tubers with traditional Nepalese starter culture, *murcha* following the same traditional way as people in normal household would do. Changes in the various parameters of fermenting mash were observed in each 3 day period.

The pH and TSS of cassava *jand* gradually reached upto 3.64 and 7 respectively when the mash was subjected for fermentation for 21 days. Protein content in the mash was found to be increased to 3.19% from small 1.84%. This apparent increase in protein may be due to the proliferation of single celled biomass.

Final alcohol content of cassava *jand* after the completion of fermentation was analysed to be 13.637% m/v.Cyanogenic glycoside content of cassava was found to be decreased significantly upto the safe levels recognised by FAO.

Thus, the traditional process of making cassava *jand* can be considered quite beneficial. Though unknowingly, this method of processing cassava has helped in enriching the nutritional quality of cassava by increasing the availability of contained nutrients, increasing the aesthetic value and the most importantly, by rendering the cassava safe from its natural toxins.

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Appendices

Appendix A

Table A.1 One way ANOVA (no blocking) for Alcohol

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days	7	626.8853	89.55504	1571.14	<.001
Residual	16	0.912	0.057		
Total	23	627.7973			

Since F pr. <0.05, there is significant difference between the samples.

Table A.2 One way ANOVA (no blocking) for Cyanogen

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days	7	5068.771	724.1101	4390.8	<.001
Residual	16	2.6386	0.1649		
Total	23	5071.41			

Since F pr. <0.05, there is significant difference between the samples.

Table A.3 One way ANOVA (no blocking) for Protein

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days	7	6.070849	0.867264	160.08	<.001
Residual	16	0.086681	0.005418		
Total	23	6.15753			

Since F pr. <0.05, there is significant difference between the samples.

Table A.4 One way ANOVA (no blocking) for reducing sugar

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days	7	388.7306	55.53294	1850.33	<.001
Residual	16	0.4802	0.03001		
Total	23	389.2108			

Since F pr. <0.05, there is significant difference between the samples.

Table A.5 One way ANOVA (no blocking) for pH

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days	7	15.6126	2.230371	1454.59	<.001
Residual	16	0.024533	0.001533		
Total	23	15.63713			

Since F pr. <0.05, there is significant difference between the samples.

Table A.6 One way ANOVA(no blocking) for TSS.

Source of variation	d.f.	s.s.	m.s.	v.r.	F pr.
Days	7	740.92	105.8457	12701.49	<.001
Residual	16	0.133333	0.008333		
Total	23	741.0533			

Since F pr. <0.05, there is significant difference between the samples.

Appendix B

Summary of ANOVA of changes in various parameters during the fermentation of cassava *jand*

Parameters	Days								LSD
	0	3	6	9	12	15	18	21	
Alcohol	0 (0)	2.43 (0.12)	5.46 (0.16)	9.32 (0.43)	12 (0.42)	13.2 (0.16)	13.7 (0.15)	13.64 (0.09)	0.413
Reducing sugar	0 (0)	1.71 (0.46)	11.87 (0.14)	6.03 (0.06)	0.56 (0.06)	0 (0)	0 (0)	0 (0)	0.299
TSS	0(1)	19.03 (0.1)	16.67 (0.21)	11.33 (0.06)	8.17 (0.06)	6.96 (0.06)	7 (0.06)	7 (0)	0.158
pH	6.11 (0.09)	4.9 (0.03)	4.12 (0.0.2)	3.8 (0.02)	3.8 (0.02)	3.8 (0.01)	3.66 (0.02)	3.64 (0.01)	0.067
Cyanogen	45.12 (0.17)	41.2 (0.76)	31.4 (0.37)	23.7 (0.41)	16.1 (0.39)	7.52 (0.45)	7.16 (0.2)	6.91 (0.1)	0.702
Protein	1.84 (0.07)	1.9 (0.02)	2.9 (0.03)	2.95 (0.05)	3.04 (0.07)	3.1 (0.02)	3.13 (0.003)	3.19 (0.17)	0.127

Appendix C

Table C.1. Standard chart for specific gravity versus alcohol content

S. G.	A. C.	S. G.	A. C.	S. G.	A. C.	S. G.	A. C.	S. G.	A. C.	S. G.	A. C.	S. G.	A. C.
0.9935	4.42	0.9625	28.70	0.9315	47.40	0.9005	61.83	0.8695	74.31	0.8385	85.35	0.8075	94.60
0.9930	4.77	0.9620	29.07	0.9310	47.65	0.9000	62.04	0.8690	74.54	0.8380	85.51	0.8070	94.73
0.9925	5.12	0.9615	29.43	0.9305	47.91	0.8995	62.26	0.8685	74.73	0.8375	85.68	0.8065	94.86
0.9920	5.49	0.9610	29.80	0.9300	48.16	0.8990	62.47	0.8680	74.92	0.8370	85.84	0.8060	94.89
0.9915	5.85	0.9605	30.16	0.9295	48.41	0.8985	62.68	0.8675	75.10	0.8365	86.00	0.8055	95.11
0.9910	6.23	0.9600	30.51	0.9290	48.66	0.8980	62.89	0.8670	75.29	0.8360	86.16	0.8050	95.24
0.9905	6.60	0.9595	30.87	0.9285	48.91	0.8975	63.11	0.8665	75.48	0.8355	86.33	0.8045	95.37
0.9900	6.98	0.9590	31.22	0.9280	49.16	0.8970	63.32	0.8660	75.66	0.8350	86.49	0.8040	95.50
0.9895	7.36	0.9585	31.56	0.9275	49.41	0.8965	63.53	0.8655	75.85	0.8345	86.65	0.8035	95.62
0.9890	7.74	0.9580	31.91	0.9270	49.66	0.8960	63.74	0.8650	76.04	0.8340	86.81	0.8030	95.75
0.9885	8.12	0.9575	32.25	0.9265	49.91	0.8955	63.95	0.8645	76.22	0.8335	86.97	0.8025	95.87
0.9880	8.50	0.9570	32.58	0.9260	50.15	0.8950	64.16	0.8640	76.41	0.8330	87.13	0.8020	96.00
0.9875	8.89	0.9565	32.92	0.9255	50.40	0.8945	64.37	0.8635	76.59	0.8325	87.29	0.8015	96.12
0.9870	9.27	0.9560	33.25	0.9250	50.64	0.8940	64.58	0.8630	76.78	0.8320	87.45	0.8010	96.25
0.9865	9.72	0.9555	33.59	0.9245	50.88	0.8935	64.79	0.8625	76.96	0.8315	87.61	0.8005	96.37
0.9860	10.11	0.9550	33.92	0.9240	51.13	0.8930	65.00	0.8620	77.15	0.8310	87.77	0.8000	96.49
0.9855	10.50	0.9545	34.25	0.9235	51.37	0.8925	65.21	0.8615	77.33	0.8305	87.93	0.7995	96.61
0.9850	10.89	0.9540	34.57	0.9230	51.61	0.8920	65.42	0.8610	77.51	0.8300	88.08	0.7990	96.73
0.9845	11.29	0.9535	34.90	0.9225	51.85	0.8915	65.63	0.8605	77.69	0.8295	88.24	0.7985	96.85
0.9840	11.68	0.9530	35.22	0.9220	52.09	0.8910	65.83	0.8600	77.88	0.8290	88.40	0.7980	96.97
0.9835	12.07	0.9525	35.53	0.9215	52.33	0.8905	66.04	0.8595	78.06	0.8285	88.55	0.7975	97.09
0.9830	12.48	0.9520	35.85	0.9210	52.57	0.8900	66.25	0.8590	78.24	0.8280	88.71	0.7970	97.21
0.9825	12.88	0.9515	36.16	0.9205	52.81	0.8895	66.45	0.8585	78.42	0.8275	88.87	0.7965	97.32
0.9820	13.29	0.9510	36.47	0.9200	53.05	0.8890	66.66	0.8580	78.60	0.8270	89.02	0.7960	97.44
0.9815	13.70	0.9505	36.78	0.9195	53.28	0.8885	66.87	0.8575	78.78	0.8265	89.18	0.7955	97.56
0.9810	14.11	0.9500	37.09	0.9190	53.51	0.8880	67.07	0.8570	78.96	0.8260	89.33	0.7950	97.67
0.9805	14.51	0.9495	37.39	0.9185	53.75	0.8875	67.28	0.8565	79.14	0.8255	89.48	0.7945	97.78
0.9800	14.92	0.9490	37.70	0.9180	53.98	0.8870	67.48	0.8560	79.32	0.8250	89.64	0.7940	97.89
0.9795	15.34	0.9485	38.00	0.9175	54.21	0.8865	67.68	0.8555	79.49	0.8245	89.79	0.7935	98.01
0.9790	15.59	0.9480	38.30	0.9170	54.45	0.8860	67.89	0.8550	79.67	0.8240	89.94	0.7930	98.12
0.9785	16.00	0.9475	38.60	0.9165	54.68	0.8855	68.09	0.8545	79.85	0.8235	90.09	0.7925	98.23
0.9780	16.41	0.9470	38.90	0.9160	54.92	0.8850	68.29	0.8540	80.03	0.8230	90.24	0.7920	98.33
0.9775	16.83	0.9465	39.19	0.9155	55.15	0.8845	68.48	0.8535	80.02	0.8225	90.39	0.7915	98.42
0.9770	17.24	0.9460	39.49	0.9150	55.38	0.8840	68.69	0.8530	80.38	0.8220	90.54	0.7910	98.55
0.9765	17.65	0.9455	39.78	0.9145	55.61	0.8835	68.89	0.8525	80.56	0.8215	90.69	0.7905	98.63
0.9760	18.07	0.9450	40.07	0.9140	55.84	0.8830	69.09	0.8520	80.73	0.8210	90.84	0.7900	98.76
0.9755	18.48	0.9445	40.35	0.9135	56.07	0.8825	69.29	0.8515	80.91	0.8205	90.99	0.7895	98.87
0.9750	18.90	0.9440	40.64	0.9130	56.30	0.8820	69.49	0.8510	81.09	0.8200	91.13	0.7890	98.97
0.9745	19.32	0.9435	40.93	0.9125	56.53	0.8815	69.69	0.8505	81.26	0.8195	91.27	0.7885	99.07
0.9740	19.74	0.9430	41.21	0.9120	56.76	0.8810	69.89	0.8500	81.44	0.8190	91.42	0.7880	99.18
0.9735	20.15	0.9425	41.49	0.9115	56.99	0.8805	70.09	0.8495	81.61	0.8185	91.56	0.7875	99.28
0.9730	20.56	0.9420	41.77	0.9110	57.21	0.8800	70.29	0.8490	81.78	0.8180	91.71	0.7870	99.38
0.9725	20.97	0.9415	42.06	0.9105	57.44	0.8795	70.48	0.8485	81.96	0.8175	91.85	0.7865	99.48
0.9720	21.38	0.9410	42.33	0.9100	57.66	0.8790	70.68	0.8480	82.13	0.8170	92.00	0.7860	99.58