

CHAPTER-I

1. INTRODUCTION

The term "Food" describes the nutritive material that may be liquid or solid consumed by human to maintain vital process, to get energy, which help in every activity of life. Among the varieties of food-"Food Beverages" is also important that involves in our daily life. Generally, alcoholic beverages are called "Bottled Beverages" and is defined as-Beverages, which are bottled and microbiologically as well as chemically controlled. Bottled beverages, may be alcoholic or non-alcoholic, carbonated or uncarbonated, an acid or non acid. (Frazier and Westhoff, 1978). Followings are the commonly used food beverages available in market:

BEER:

Beer means alcoholic beverage i. e. contains 2% or more than 2% ethyl alcohol by volume which is properly sealed in container (bottle or can) in a manner that ensures free from chemical and microbial spoilage. Beer is fermented product that is manufactured by microbial activity. Beer is also called "malt beverages". It is made of malt, hops, yeast, water and malt adjunct. Characteristics feature of the beer is: ethanol = 2-6.5%, pH = 3-4.8, carbon dioxide 0.3% (NS: 23/2040)

WINES:

Wines are essential product of fermentation of hexose sugar of fruit by intact yeast cells to form ethyl alcohol and carbon dioxide. There are three main types of wine: a) table wine b) sparkling wine and c) fortified wine.

Consumption of alcoholic beverages has increased in recent years. Such beverages are stored for many days, weeks, months. It may be assumed that, such products are more likely to be involved in food borne outbreaks and other diseases. Microbial contaminated beverages may act as vehicle for disease like gastroenteritis, enteric fever and sore throat.

The ingredients of beverages not only affect the suitability for microbial growth but can also affect the kind and number of micro-organisms present. Hence, the likelihood of spoilage organisms being added. In addition, the bottles and closures are the other possible sources of contamination. Similarly, water is another main source of contamination. Similarly, water is another main source of contamination. Hence, the water for beverages should be purified. Yeast, chiefly *Torulopsis* sps. and *Candida* sps. are most likely causes of spoilage of soft drinks. Fruit concentrates are other source of yeast.

Cloudiness and ropiness are spoilage types of soft drinks. Cloudiness results from marked growth of various yeast or bacteria and ropiness from the development of capsulated bacteria, like *Bacillus* sps.

Spoilage of beer is caused by yeast and bacteria. The spoilage patterns of beer may be classified as follows i.e. ropiness, sarcianae sickness, sourness and turbidity. The causative agents for the spoilage are *Acetobacter* sps., *Lactobacillus* sps. *Pediococcus* sps. etc, and turbidity, by *Achromobacter anaerobium* and *Saccharomyces* sps.

For *raksi*, it is obvious that a yeast culture will always be contaminated with other organism, which ordinarily include bacteria and wild yeast, that build up during successive fermentation and recoveries which may be causative agent for spoilage of *raksi*.

Such spoilage type of organism may be harmful for human being and may bring different kinds of disease like food poisoning, typhoid fever, diarrhoea. These types of disease are more prevalence in Kathmandu city. According to the Health Ministry, His Majesty's Government -the percentage of gastroenteritis is 76.3% and enteric fever is 5.4% in 1991-1992. The role of such beverages in causing these diseases could not be ignored.

Similarly, chemical parameters like: pH, acidity, alcohol content, dissolved carbon dioxide, plus organic and inorganic compound present within beverages are important quality parameters of these beverages. Trace metals like: As, Cd, etc. are carcinogenic. Chemical parameters above the recommended level may

bring chemical changes, which may be harmful to human consumption. Hence, chemical parameters also determine the quality of alcoholic beverages.

In Nepal, consumers are not very conscious about quality of such beverages and people have little knowledge on food hygiene to determine the microbial and chemical quality of alcoholic beverages of Kathmandu city. The study tried to analyze risk of consumption of such beverages associated with it. This research will be the first of its kinds in the context of Nepal.

Food hygiene depends on personnel hygiene, cleanliness of surrounding and handling of food etc. In fact food hygiene is actively designed for public protection. Hence, food hygiene has public health aspect in all its stages from production, processing, storage, distribution, marketing and consumption of finished product.

In surveillance study, hygiene aspects of such beverages were studied. People who are familiar about the contaminants of such type of food as well as harmful aspects about out break of disease through alcoholic beverages are very rare.

CHAPTER-II

2. OBJECTIVE OF STUDY

2.1 GERNERAL OBJECTIVE

The general objective of this study is to determine the microbial quality and physical parameters of some common alcoholic beverages of Kathmandu Valley. The sampling was designed to survey the microbial load and parameters of:

- a) Beer
- b) *Jand*
- c) *Raksi*

2.2 SPECIFIC OBJECTIVE

The study includes following specific objectives.

- a) To enumerate and isolate microbial load of alcoholic beverages
- b) To determine parameters of alcoholic beverages such as temperature, pH, acidity, Brix and alcohol percentage
- c) To study about the source of contamination.
- d) To provide a frame of the quality of such alcoholic beverages in Kathmandu Valley.

CHAPTER-III

3. LITERATURE REVIEW

Alcoholic beverages are the products of food. Alcoholic beverages include beer, *raksi*, *jand* etc.

3.1. HISTORY OF ALCOHOLIC BEVERAGES

The production of alcoholic drinks was started at different time, which is defined separately.

3.1.1 History of Alcoholic Beverages

BEER

With some reservation historians believed that beer production, which involves hydrolytic breakdown of starch in cereal extracts was invented in Egypt, some 500-600 year BC (Corran, 1975).

The concept of barley specifically designed for malting is a relatively recent one; though different types of barley are recommended different qualities of beer is early Babylonian texts.

Although the origins of brewing are lost in prehistory, there is little doubt that barley, the principal ingredient of beer throughout the ages, is one of the oldest man's cultivated plants (Zohary, 1969).

Beer is an alcoholic beverages made from fermented malt, flavoured with hops etc. has the merit being comprehensive it names, directly or implication, the four essential normal contributors to all types of beer normally water, malt, hops and yeast considerable scope for the inclusion of other ingredients.

Certainly, the case of crushed malt which was crumbled into water to provide either nutrition's gruel or a fermentable extracts is well attested in early Egyptian records (Wild, 1966).

Corran, (1975) has confirmed that the feasibility of baking malt bread at Egyptian summer temperature and subsequently fermenting the mash made by crumbling the loaf into warm water, the resulting unfiltered beer contained 3-5% of alcohol.

In 1680, Dutch scientist Antony van Leeuwenhoek was the first person to see yeast cells when he examined drops of fermenting beer with his primitive microscope. Later on the concepts of fermentation was advanced by three workers Cagniard Latour, Schwann and Kützing (1837), mainly on the basis of microscopic examination of fermenting liquids (Chapman, 1931).

Interestingly, the brewing process in its early states of development, resembled the baking of bread, barley was placed in an earthenware vessel and buried in the ground until germination began. Then it was crushed made into a dough and baked until a crust formed. Finally the dehydrated dough was soaked in water until fermentation was completed. The acid beer so produced was called BOOZAH., a term that is now used in quite different sense in Britain to describe the location of beer drinking rather than the product (King, 1947).

WINE

Exploiting the ability of certain yeast to produce potable and stimulating beverages by alcoholic fermentation dates to very early times, certainly to several thousands of years BC. It is surprising to discover that the first occasions on beverages resemble what we now know as beer, cider and wine were made but was not documented (Hardwick, 1975).

In likelihood, the first wine was made from fruit possibly grape juice quite inadvertently, since juices extracted from most types of fruit are contaminated with microbes, including yeast, that from the surface flora or bloom of the fruits, with the result that the juices are susceptible to alcoholic fermentation provided that the environment temperature is conducive. Nor can it be stated where the first wine were made, although many authorities believe that it was probably in the valley of what we know as the River Tigris in Iraq.

In 1837, Schwan dubbed yeast the causative agent "Zuckerpilz" from which the name *Saccharomyces* or "sugar fungus" originates.

The microbiology of brewing made a significant advanced over the microbiology of wine making when, starting around 1880, the Danish botanist Emil Christian Hansen began publishing some epoch-making investigation into the morphology and physiology of yeast (Jorgensen, 1948).

The history of distilling of potable spirits is shorter than that of making non-distilled alcoholic beverages. There are nevertheless several very early references which can be taken to indicate that a potable spirit was known many thousand of years ago. The earliest reference to the production and the official warning regarding excessive consumption of potable distilled spirit appear to have come in China in 1000 year BC (Legge, 1983).

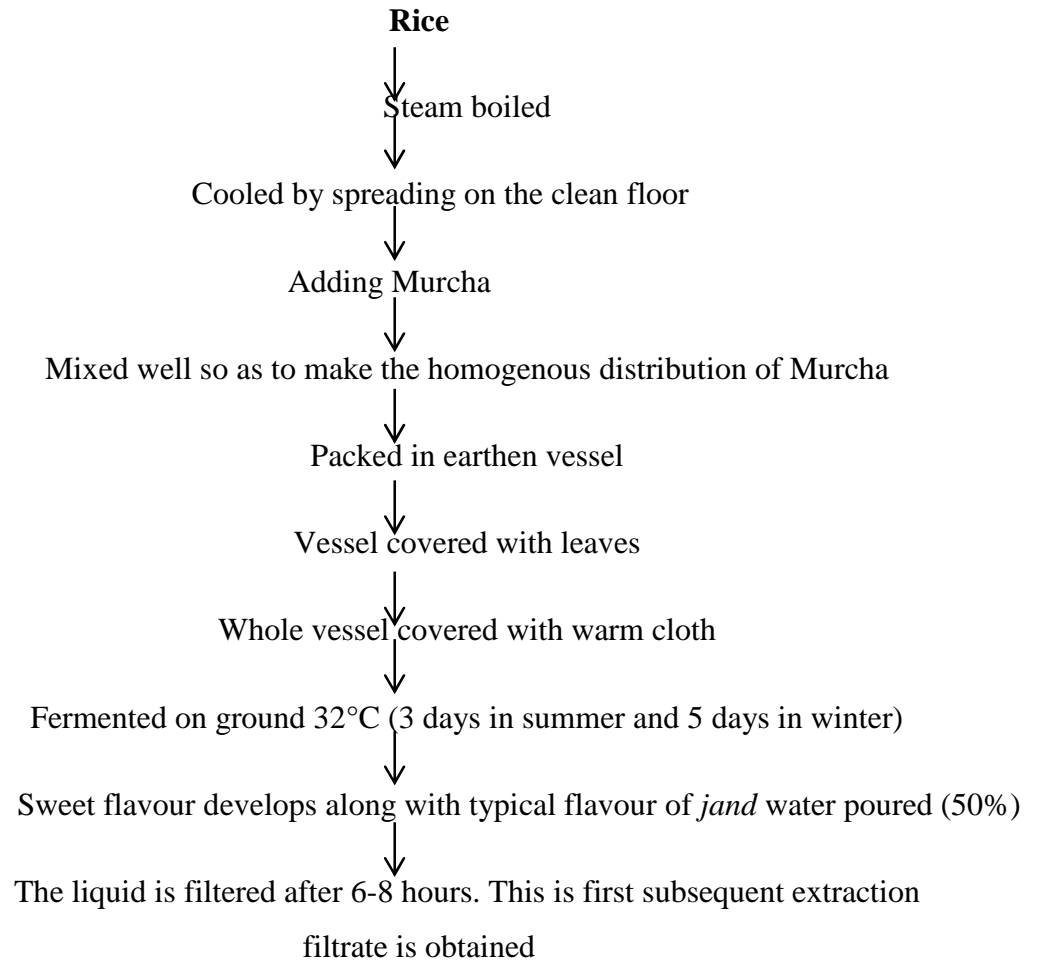
Raksi is fermented product using yeast and mold as inoculum and rice, maize, millets as substrate and are distilled beverages containing 20-40% of alcohol (Karki, 1986).

JAND

The exact period of these products is not known. In Nepal, it is said that the custom of worshipping gods and goddess was by Tantric process and alcoholic beverage were offered during worship. This indicates that the existence of alcoholic beverage has a long tradition in Nepal (Karki, 1986).

Jand is beer type of product which is fermented from rice and maize using the yeast and mold which is undistilled fermented product containing 6-10% of alcohol. (Karki, 1986).

Flow Sheet for the production of *Jand* in Nepal



3.2 ALCOHOLIC BEVERAGES IN NEPAL

In Nepal there is no long history of production of alcoholic beverages. Beer is the first alcoholic beverages produced in manufacturer's level, though production of *jand* is produced traditionally from very early days in most of the lower cast.

3.2.1 Beer in Nepal

In 1977, Nepal Brewery (Pvt) Ltd. produced its first product in market, as "Star Beer" hence the production of beer started in Nepal since 1977. Nowadays, in Nepali market seven different types of beers are found. Most of them are being manufactured in collaboration with renowned international companies. Himalayan Brewery is second beer manufacturer in Nepal as "Iceberg beer. Thereafter, San Miguel Beer is product of Mt. Everest Brewery since 1990, which is under the supervision of San Miguel Brewing International Ltd. Golden Tiger Beer is another product of Mt. Everest Brewery. Other popular brands are Tuborg and Carlsberg by Gorkha Brewery Ltd. recently, Chowdhary group of industry launched another beer named Singha. Almost all the beer are lager beer. The Beer companies established in Nepal are shown if following table with production date.

| Manufacturers | Products Name | Established |
|----------------------------|--------------------------------------|------------------------|
| Nepal Brewery (P). Ltd. | Star Beer | 1977 |
| Himalayan Brewery (P) Ltd. | Iceberg Beer | - |
| Mt. Everest Brewery Ltd. | San Miguel Beer Golden tiger Beer | 1990 |
| Gorkha Brewery Ltd. | Tuborg Beer Carlsberg Beer | 1991-1992 1994-1995 |
| Chowdhary Group | Singha Beer | 1996 |

3.2.2 *Jand* and *Raksi* in Nepal

Alcoholic beverages are prepared throughout the country. In Nepal *raksi*, is considered as one of the essential components of the meal in some communities. The process is inherited traditionally and is passed on from generation to generation. Alcoholic beverages are practiced in Nepal since long time. The exact period of origin of *jand* is not known. In Nepal it is said the custom of worshipping gods and goddess was tantric process and alcoholic beverages were offered during worship. This indicated that the existence of alcoholic beverages has a long tradition in Nepal. *Jand* is beer type product prepared from boiled rice and other cereals.

3.3 ALCOHOLIC BEVERAGES AND IT'S TYPE

Beverages which are made from food either fermented e.g. beer or non-fermented fruit juices, carbonated drinks which are bottled and microbial plus physico-chemical parameters and controlled. Bottled beverages may be alcoholic or non-alcoholic, carbonated or uncarbonated, an acid or non acid. Broadly, types of beverages are following types.

3.3.1 Alcoholic Beverages

BEER

Beer means alcoholic beverages i.e. contains 2% or more than 2% ethyl alcohol by volume which is properly sealed in container (bottle or can) in a manner which ensure free from chemically and microbial spoilage. The main type of malt liquors can be classified as follows.

- a) **Bitter or Pale ale:** a high quality product containing then most hops.
- b) **Mild ale:** which is sweeter and contains less bitter
- c) **Stout :** brewed mainly in Dubling and London which is dark in color due to the used of caramelized and black malt.

- d) **Lager** : Which is brewed more especially on the continent by bottom fermentation and tends to contain more extractive and less alcohol than other types. In Nepal only larger beer is available.

WINE

Wine is essential product of fermentation of hexose sugar of fruit (usually the grapes) by intact yeast cell to form ethyl alcohol and carbon dioxide. Following are main classes of wine.

- a) Table wines containing 6-15% V/V alcohol produced by spontaneous fermentation due to organism on the fruit surface e.g. Burqundy, Claret, Hock etc.
- b) Sparkling wines which are given a second fermentation in the bottle so as that a visible excess of carbon dioxide is produced e.g. Champagne. With inferior type carbonation's replace the secondary fermentation.
- c) Fortified wines containing about 20% v/v alcohol made by the addition of spirits to wines e.g. port sherry, Madeira.

Wine can be further sub classified according to area, variety of group used, colors and degree of sweeteners (Marrison, 1959)

JAND

It is Nepali traditional alcoholic beverages, which is beer type product prepared from boiled rice. The filtrate after fermentation with murcha is called *Jand*. Murcha is essential ingredient to start the fermentation. *Jand* is undistilled type of alcoholic beverages. The distillate from *jand* is called *raksi*.

3.4 MICROBIOLOGY AND BIOCHEMISTRY OF ALCOHOLIC BEVERAGES

Alcoholic beverages which are distilled to yield potable spirits have in common that they are produced by the alcoholic fermentation of sugar containing nutrient solution by species of the yeast *Saccharomyces*. The source of sugar in

the liquid varies with the nature of the beverages. With some, such as wines made from grapes or other fruits the sugar is present as such in the raw material. In other, it is formed following the hydrolysis of sugar of polysaccharides in the raw material. The availability of free sugar for alcoholic fermentation by species *Saccharomyces* is essential. Since, none of the species that are used to ferment alcoholic beverages has the capacity to produced extracellular enzyme capable of catalyzing hydrolysis of polysaccharide. The genus *Saccharomyces* is just one of the 39 genera of single celled fungi or yeast recognized in standard taxonomic treatise on yeast. (Lodder, 1970)

Throne (1970) has developed a scheme by which brewing yeast can adequately and usually describe in term of the following characteristics, rate and extent of growth, rate and extent of fermentation, flocculance, flavour and aroma of the beer produced.

Saccharomyces spp. as a wine yeast, this important species is known as *S. cerevisiae* var. ellipsoids. But recent revision of the taxonomy of yeast have eliminated it as separate variety. (Van der Walt, 1970).

Schanderl (1959) reports on the selection of strains of *S. cerevisiae* with the following characteristics:

- High ethanol production of 18-20% of alcohol
- Cold resistance
- Alcohol tolerance
- Osmotolerant etc.

Microorganism associated with fermentation were *Lactobacillus plantarum*, *Pediococcus acidolactic*, *L. fermentum* and *S. cerevisiae*. During fermentation, the temperature and moisture increased and pH decreased. Most amino acid decreased in concentration during fermentation with glutamic acid being the most abundant amino acid but the protein content increased during fermentation. The bacteria played the major role in the fermentation of alcoholic beverages (Rose 1977).

In some wine producing countries such as USA, Canada, New Zealand and Australia and more recently in European countries selected yeast culture are inoculated into grape juices to induce fermentation. It is believed that the inoculated species usually single strain of *S. cerevisiae* dominates fermentation. The yeast directed fermentation in pure culture are more efficient, regular and continuous (Amerine and Junkee, 1968).

3.5 CHEMICAL PARAMETER OF BEVERAGES

3.5.1 Alcoholic Beverages

A complete analysis of wine should include the estimation of specific gravity, alcohol, ash, acidity, sugar, glycerol, sulfur dioxide, sorbic acid and possibly an examination for other like preservatives, added dyes, methanol, isopropanol and artificial sweeteners, these are seldom sampling problem with ordinary types but sparkling wine and other showing froth should be de-gassed. Method used for the analysis of wines have been reviewed by Amerin and Ough (1972).

It has been agreed that the international system of expressing alcoholic strength should be as the percentage of alcohol by volume at 20°C (Savage, 1973).

Cooke (1974) has shown that it is possible to obtain the alcoholic strength from values of their refractive indices and relative densities at normal ambient temperature.

Dixon *et al.*, 1963 have designed a field test for obtaining the spirit strength of wine. Acidity of wine calculated as milliequivalent per litre European Economic Community (EEC) or as tartaric acid. The total tartaric acid present in wine may vary from 0.07-0.5%. The standard EEC method involves isolation by anion exchange column of the tartaric acid, which is then determined colorimetry by means of red color formed with vanadic acid (Pearson, 1976).

The estimation commonly required to be carried out on beer are those for specific gravity, alcohol, preservatives, arsenic and lead. For the discussion of these and other methods which have been proposed the reader should refer it.

The publications of the Institute of Brewing Analysis Committee (1971) and the earlier historical review of Hudson (1960).

Various analytical methods have also been recommended by the European Brewery Convention. The analysis of alcohol of beer sample anytime is found to be of an original gravity not exceeding one thousands and sixteen degrees and to be of a strength not exceeding two degree of proof (Custom and Excise Act section 307 1952).

pH

The excellent keeping quality of food beverage is relative to their restrictive pH. Food and food products with low pH (Below 4.5) usually are the not readily spoiled by bacteria and are more susceptible low pH would therefore tend to be more stable microbiologically than a neutral food. Fermented product has low pH because of developed acidity form the accumulation of lactic acid during the fermentation.

Not only are the rates of growth of microbes affected by low pH but so are the rates of survival during storage and other forms of processing. Initial pH may change later due to growth of micro-organism which may be more favourable range for the growth of other micro-organism (Frazier and Westhoff, 1978).

3.7. MICROBIOLOGICAL SPOILAGE OF BEVERAGES AND THEIR EFFECT

3.7.1 Spoilage of Alcoholic Beverages

Pasteur (1866) noted that wine is most hygienic of all beverages. The very few spoilage organisms which can survive in wine may be unpleasant but not unhealthy. Wine has always been considered a safe and healthy beverages. It has been said water separates of the worlds but wine them (Gates, 1971).

It is certainly true that the industrial, scientific and social revolution have brought with them an internationalization of wine usage and democratization of wine (Lausanne, 1970).

Some lactic acid bacteria are surprisingly 'at home' contaminate wine. In spite of low pH value, high concentration of ethanol, low storage temperature and sulfur dioxide, strain of *Leuconostoc*, *Lactobacillus* and *Pediococcus* will grow in wine albeit slowly and induced malo lactic reactic reaction which involves decarboxylation of malic acid to lactic acid. There are three aspects for the conversion of malic acid to lactic acid

- 1) Loss of acidity due to decarboxylation
- 2) Change in the flavour of the wine resulting from the by-products of bacterial fermentation.
- 3) Microbiologically stability following growth of organism particle information on how to induce the malolactic fermentation, including inoculating with *Leuconostoc oenos* (Kunkee, 1974).

Malo- Lactic bacteria may add complexity of flavour to the wine by formation of end product, some at low concentration (Pilone, 1975)

Flavour change brought by the deacidification, but only further change or improvement in flavour by increasing the complexity of compound present (Singleton and Ough, 1962).

Amerine (1972) noticed that the consumer demands for more cleanliness and with the advanced in sanitary engineering which made cleanliness relatively easy wineries and dairies have had to present themselves. Lactic acid bacteria are the only bacteria which will grow on properly stored table wine, that is table wine kept under anaerobic condition and with or measurable concentration of free sulphur dioxide. The cotton mold or *Bacillus*, *Lactobacillus trichodes*, is a spoilage organism of wine.

Spoilage by acetic acid bacteria will not be common in anaerobically stored wine i.e. wine in bottles with tight wet corks or screw capes or in tanks blanketed with an inert gas such as carbon dioxide, nitrogen.

Yeast such as, *Candida* forms film that have little effect on flavour of wine. The *Mycoderma vini* films should not be confused with *Mycoderma aceti*, the old

name for film forming acetic acid bacteria. Strains of *Pichia* have also been reported as surface contaminants of stored wine.

Another example of yeast spoilage is that caused by *Brettanomyces* spp. Problem with this organism is seldom, but the astute wine maker should be familiar with its appearance effect and control (Van der Walt and Van Kerkeken, 1958, 1961, Vanzyl, 1962). The spoilage can often be noticed by metallic character imparted to the flavour of wine. Perhaps the most serious yeast spoilage of wine should be by *Saccharomyces ludwiggi*, since this organism has been found in wine made from highly sulphited results. (Amerine and Kunkee, 1968). Three types of yeast sps which have been said to cause this kind of spoilage of wine are the *Candida rugosa*, *Pichia membranefaciens*, *S. acidifaciens*, *S. bayanus*, *S. cerevisiae*, *S. chevalieri* and *S. uvarum*. (Scheffer and Mark, 1951; Vander Walt and Van Kerken, 1958).

Brettonomyces sps. already mentioned in connection with spoilage of bulk wine have, sometimes, been the causative agents of bottled wine spoilage. (Vander Walt and Van Kerkeken, 1958). The most common defect of wine is acidification. This is caused by *Gluconobacter* and *Acetobacter*. All alcoholic beverages containing less than 15% w/v ethanol are subjected to this type of spoilage. Species of *Leuconostoc* and *Streptococcus* (especially *Leuconostoc mesenteroids*) as well as some *Acetobacter* can produce dextrans in sweet wine. In this case, a ropy texture is develop which then becomes slimy and viscous, Types of defects in wine are acetification, mousy odor, cloudy, slimy by *Acetobacter*, *Gluconobacter*, Lactic acid bacteria respectively. Acetaldehyde by *Saccharomyces oriformis* and *S. beticus*, wine flowers by *Candida vini* (Banwart, 1987).

Wine has its defects from microbial causes are chiefly wild yeast, molds and bacteria of genera *Acetobacter*, *Lactobacter*, *Leuconostoc* and perhaps *Micrococcus* and *Pediococcu*. (Frazier and Westoff, 1992).

Acetobacter sps. can make their own vitamins but the lactic acid bacteria must have most of provided. The chief sources of these substances in wine is the wine yeast, which release the necessary growth factors on autolysis. The move

of these substances there are present greater the likelihood of spoilage by lactic acid bacteria (Frazier and Westhoff, 1978).

Film yeast, which can oxidize alcohol and organic acid, may grow on the surface of the must and wines exposed to air producing a heavy pellicle called “wine flower”. They should cause not trouble if the must is mixed periodically and if air is kept away from the wine. *Candida mycoderma* is important type of yeast for the wine flower (Hobbs, 1982).

In the presence of air the aerobic acetic acid bacteria, usually *Acetobacter aceti*, *Gluconobacter oxydans*, oxidize alcohol in must or wine to acetic acid an undesirable process called acidification. They also may oxidize glucose in the must to gluconic acid and may give a “mousy” or “sweet sour” taste to must.

Molds such as *Mucor*, *Penicillium*, *Aspergillus* and other, may grow on plant walls, barrels, tanks, hoses and corks and may also grow on the grapes or on cold must. Molds are kept down by adequate cleansing of wall and equipment. Spoilage by facultative micro-organism, wild yeast, which includes all yeast but wine yeast added as starter, may bring about abnormal fermentation that result in low alcohol content, high volatile acidity, undesirable flavours and cloudiness in the wine. These yeast which come from the grapes, from the must is prepared and usually are predominately of the aciculate type are suppressed or eliminated by the use of an active starter of wine yeast, sulfating or pasteurization of the must before the fermentation and control of the temperature of must during the fermentation. Low temperature, below 21⁰ C will favor the growth of some of the wild yeast and slime-producing bacteria.

Lactic acid bacteria are the principal causes of bacterial spoilage of musts and wines. There have been some confusion in the application of names to the various types of bacterial spoilage of wines, probably because different kinds of bacteria may be able to cause the same defect or because the same organism may cause different defect under different conditions. Probably most occurring is **tourne** (turned or soured) spoilage, in which acid is formed from sugar, glucose and fructose in the wine, chiefly by heterofermentative *Lactobacillus* species such as *L. hilgardii*, *L. trichodes* and perhaps *L. buchneri*. The growth

of *Lactobacillus* produces silky cloudiness increase lactic and acetic acid, yields carbon dioxide sometimes gives mousy or other disagreeable flavours and damages the color of wine. Gassiness resulting any causes, such as from liberation of carbon dioxide by heterofermentative lactis, is called pousse. The homofermentative *L. plantarum* forms mostly lactic acid from sugar in table wines, increasingly the fixed acidity and giving a mousy odor (Jay, 1987).

The acidity of wine may reduced by the spoilage bacteria through the oxidation of the malic, lactic and tartaric acid by species of *Lactobacillus*, *Leuconostoc* or *Pediococcus* or by other other cocci.

Sliminess or ropiness of young white wines accompanied by cloudiness and increased volatile acidity has been blamed on *Leuconostoc* sps. *L. mesenteroides* and *L. destranicum* or *Micrococci* and *Lactobacilli*. Addition of sucrose, when permitted favors the production of dextrans and therefore of sliminess by *Leuconostoc* spp.

Effect of storage temperature (room 30⁰ C and refrigerator 4°C) on microbial counts was investigated. Storing the products at room temperature markedly increase in population densities of all examined microorganism. However, refrigerator temperature should or slight increase in total microbial counts (Daw, 1994).

3.7.2 Spoilage of Beer

Owing to its low pH value (about 4.0), beer is an inhospitable medium for most bacteria though many wild (i.e. non culture) yeast can thrive in it when Pasture (1879) examined pitching yeast, his observation doubtless fascinated and possibly horrified the brewers concerned, but whether his observation on the acute power of diseased ferments was acted on his another matter, Certainly, 60 years later, Shimwell. (1936) developed the fact that there was not a single beer disease organism in the British National Culture Collection, an omission which he soon rectified by depositing there in culture of *Lactobacillus pastrorians* and *Acetobacter capuslatum* (now *Acetomonas oxydans*) though tribute is always and slightly paid to Pasteur scientific brewing bacteriology really began with

Shimwell, the acetic acid bacteria (*Acetobacter* spp. and *Acetomonas* spp.) are still probably the commonest beer spoilage organism, and to them must be added *Zygomonas anaerobia*, also isolated from beer (Shimwell, 1937).

Since the acetic acid bacteria are aerobic their growth is restricted on the surface of beer and result in the development of a characteristics vinegary aroma and flavour. *Acetomonas oxydans* which can tolerate very low levels of oxygen, produces gelatinous capsule, which to the naked eye, appear as voluminous “ropy” strands; this objectionable slimy material is formed from the beer dextrin’s (Shimwell, 1947a)

Brewer *Lactobacilli* such as *lactobacillus pastorianus* or *L. lendneri*, the latter of which according to Ault (1971), is common in larger fermentation are more troublesome than the acetic acid bacteria because they are facultative anaerobes. They are nutritionally fastidious and many beers contain sufficient residual aminoacids to support their growth. Some lactic acid forming cocci (*Pediococcus damnous*) also produced large amount of diacetyl, so giving rise to turbid acid evil swelling beer. None of these organism should survive pasteurization. Finally, the soil organism *Zygomonas anaerobia* which produce acetaldehyde and hydrogen sulphide during fermentation of glucose to give swell reminiscent of rotten parsnips may gain access to primed cask beer during conditioning or in trade, and cause spoilage within 2-3 days. (Dadds and Martin, 1973).

In recent years, wild yeast which are capable of growing in beer to produce turbidity have increasingly drawn attention as beer spoilage organism.

The Industrial spoilage of beer and ales is commonly referred to as **beer infection**. This condition is caused by yeast and bacteria. The spoilage patterns of beer and ales may be classified into four groups: Ropiness, Sarcinae sickness, Sourness and Trubidity.

Ropiness is a condition in which the liquid becomes characteristically viscous and pours as an “oily” stream. It is caused by *Acetobacter*, *Lactobacillus*, *Pediococcus cerevisiae*, and *Gluconobacter oxydans* (formerly *Acetomonas*).

Sarcinae sickness is caused by *P. cerevisae* which produced a honey like odor. This characteristics odor is the result of diacetyl production by the spoilage organism in combination with the normal odor of beer.

Sourness in beers is caused by *Acetobacter* spp. These organisms are capable of oxidizing ethanol to increase levels of acetic acid.

Turbidity and off odor in beers are caused by *Zygomonas anerobia* (formally *Achromobacter anaerobium*) and several yeast such as *Saccharomyces* spp. Growth of bacteria is possible in beers because of a normal pH range of pH range of 4.5 and a good content of utilizable nutrients.

An interesting gram negative anaerobes rod was isolated (Lee et. al. 1978) at the brewer in Colorado from turbid beer and placed in the family *Bacteroidaceae*. Classified as *Pecitnaus cereniiphilus*, some has been also reported from breweries in Germany and Finland.

With respect to spoilage beer, one of the major contaminants found is *Saccharomyces diastaticus*, which is able to utilize dextrans that normal brewers yeast cannot. *Pediococci*, *Flavobacterium*, *Proteus* (formally *Obesumbacterium*) and *Bretannomyces* are sometime found in spoiled beer.

Data are given for the frequency of occurrence of spoilage bacteria (*Lactobacillus*, *Pedeococcus*, *Pectinantus megasphaera* spp. and *Enterobacteriaceae*) in 148 samples of spoiled beers *L brevis*, *L. lindneri* and *Pediococcus damnous* were the most frequent species. *Magasphaera* and *Pectinatus* species are of increase importance. Infection of beer via raw materials or secondary is discussed. Main route of infection for the individual species are considered (Back et.al., 1987).

A new *Lactobacillus* spp. *L. brevisimilis* isolated from a range of spoiled beer is described. Morphological, biochemical, and physiological characteristics are discussed and differentiation from *L. brevis* and *L. lindneri* considered (Peischmann, 1988).

Wiles, (1953) described classical methods suitable for identification of such infecting organism but for rapid control immunofluorescent techniques. Richard, (1969) has developed this technique to a point at which are "wild" *Saccharomyces* cell per million culture cell can be detected and, as Ellison and Doran (1961) has found that order of contamination sufficed to produced a haze by non fining *Saccharomyces cerevisiae* the technique the technique is clearly an useful one.

One of the obnoxious wild yeast contaminants is *Saccharomyces diasticus*. This yeast can ferment the dextrin of beer and simultaneously produced off-flavor, one cell per bottle is enough for bottle.

Fermentation may be responsible for cloudiness. Wild yeast e.g. *Saccharomyces pastorianus* can cause cloudiness in beer (Frazier and Westhoff, 1978).

Streptococcus mucilaginous which probably is a *Pediococcus* has been reported to cause (Frazier and Westhoff, 1978).

Lactobacilli sps. are hops tolerant. These organisms produced lactic acid dextrans hazed and dizcetyl in beer. A diacetyl flavour generally is not desirable in the light larger beers produced in USA. Several yeast such as *Brettanomyces*, *Candiada* and *Pichia* can infect finished beer and with secondary fermentation causes defects (dextrans hazes films off flavour and off-odor) in the beer.

The decarboxylation of *hydroxycinnamic* acids normally present in barley, by certain *enterobacteria*, such as *Hafnia*, a result in a phenolic off-flavour in beer (Lindray and Priest, 1975).

3.7.3 Microbial Spoilage of Jand

Murcha is a traditional starter, used commonly in Darjeeling hills and Sikkim in India to ferment a variety of starchy substrate in order to produce sweet-sour alcoholic beverages called *jand*. Murcha cakes are mildly acidic (pH 5.2) and contain 13% w/w moisture and 0.7 w/w ash. A total of 194 bacterial, 190 yeast and 80 molds strains were isolate from 30 samples of murcha. The counts cfu/g fresh weight of micro-organism in sample were 2.0×10^7 to 4.2×10^8 for

Pediococcus pentosaceus 4.0×10^7 to 6.8×10^8 for *Saccharomycopsis fibuligera* 2.0×10^6 to 7.2×10^7 *Pichia anomala* 1.0×10^6 to 4.1×10^7 for *Mucor circinelloides* and, <10 to 1.0 for *Rhizopus chensis*. Which all the species were prevalent in 100% of sample. They may bring undesirable change on product (Tamang and Sarkar, 1995).

CHAPTER-IV

4. MATERIALS AND METHODS

Three different types of alcoholic beverages were collected randomly from retail shop of Kathmandu city (Dec. 2002 to Dec. 2003) and were analyzed microbiologically as well as chemically.

The purpose of study designed as the alcoholic beverages found in Kathmandu valley were suitable for consumption or not. Besides that the total load of bacteria and yeast and molds were also determined and chemical parameters were also checked.

4.1 SAMPLING SITES

The survey was conducted in Kathmandu valley i.e. include Kathmandu, Bhaktapur and Lalitpur. During the survey maximum of five samples of each beverages were taken. This study covered the three different types of alcoholic beverages. Every retailer was asked about time when did they collect from the manufacturer and try to note down the manufacture dates. The sampling sites were three districts of Kathmandu valley. Retail shops were selected randomly for sampling (Annex-I). It is important that samples of alcoholic beverages collected for study accurately reflect microbiological condition of the time of sampling.

4.2 SAMPLE COLLECTION

Random sampling procedure was adopted for sample collection. While sampling, all extraneous contamination were avoided and delivered to the laboratory. Samples were kept in the refrigerator until analysis. Samples were analyzed within an hour of arrival as described by manual of food quality control, microbiological analysis.

4.3 PROCESSING OF SAMPLE

Samples were processed in following ways, which were described separately.

Alcoholic beverages were purchased from retail shops. The bottles were cleaned externally with absolute ethanol to disinfect and samples processed using two distinct methods i.e. Pour Plate Technique and MF-technique.

4.3.1 Serial Dilution

Samples were diluted separately and aseptically in a series of tubes containing sterile 9 ml of water. Samples were diluted upto 10 dilution. 1 ml of sample was pipetted aseptically and poured to tube containing 9 ml of sterile water, shook well and labelled 10^{-1} ml from 10^{-1} dilution to next tube containing 9 ml sterile water and shook well which gave 10^{-2} dilution. The process was repeated for 4th, 5th, 6th, 7th, 8th, and 10th tube which gave were respective dilution (Ogg and Sharma, 1977).

4.3.2 Pour Plating

Pour plate method consists of diluting out organism in such manner that one of the plates poured will have an optimum number of organism for isolation, as well as serial dilution was not done for some sample to check out the microbial load presence in given sample.

4.3.3 Membrane Filter Technique (MF-Technique)

This method aseptically suited for samples that contain low number of bacteria. 100 ml of each sample is poured into sterile Millipore Filter assembly and allowed to pass through membrane filter by the help of vacuum pump or aspirator that will retain the micro-organism then membrane was placed on an agar plate with culture medium of choice and incubated appropriately. Sterile membrane was used and aseptic technique was applied. From each alcohol cleaned bottle 100 ml were removed and filtered either through a 0.4 μm -pore membrane filter for bacteria.

4.3.4 Incubation of Membrane Filter

Following the collection of microorganism upon filtering a given volume, the membrane was placed on an agar plate or an absorbent pad saturated with

culture medium of choice i.e. media used were plate count agar for total load of bacteria, violet red bile agar for coliform count and potato dextrose agar for yeast and mold count.

In case of membrane filter technique-only small volume of sample can be used for single membrane.

4.3.5 Total Bacterial Count

Total bacterial count was performed according to the standard plate count technique which essentially consists of following steps:

- i. One ml. of dilution of sample was pipetted out and kept into each of sterile appropriately marked duplicates plates.
- ii. Sterilized plate count agar was cooled down to 45⁰ C and about 15 ml of molten agar was poured into each petridish.
- iii. Sample dilution and agar medium was mixed thoroughly and uniformly. It was then allowed to solidify.
- iv. Solidified plates were incubated inverted at 37°C for 48 hours.
- v. All colonies on dishes containing 30-300 colonies were counted and results were recorded per dilution. The results were expressed in terms of colony forming unit per ml of samples (cfu/ml).

4.3.6 Enumeration of Yeast and Mold Count

Samples which were diluted were kept in Potato Dextrose Agar (PDA) and after solidification were incubated. In case of membrane filter technique, the membrane was placed aseptically in solidified media and was incubated at 25°C for 48 hrs. Similarly, results were expressed in cfu/ml.

4.4 METHOD ON ANALYSIS OF ALCOHOLIC BEVERAGES

The analysis of the alcoholic beverages may includes the determination of the following parameters i.e. temperature, pH, acidity, total soluble solids (Brix), alcohol and percentage.

4.4.1 Temperature

To measure the temperature, simply sensitive thermometer was used. Thermometer was dipped into sample containing vessel for 2-5 minutes and thermometer reading was recorded (Annex-1).

4.4.2 pH

The most convenient and reliable method for measuring pH was by using pH meter, which measure e.m.f of a concentration cell formed from a reference electrode. Followings are the steps used for the measurement of pH.

- i. First of all, pH meter was calibrated by using standard solution having known pH.
- ii. Then, pH meter submerged into sample. The readings were noted.

4.4.3 Acidity (Percent of Citric Acid or Acetic Acid or Tatrtritic acid)

For routine purpose, it is usually sufficient to determine the titrable acidity (using phenolphthalein). Following is the methods according to AOAC for the determination of acidity percentage.

- i. 10ml of sample was pipetted into clean beaker
- ii. Phenolphthalein was added as indicator
- iii. Then, sample was titrated with 0.1M of NaOH till slight pink color was appeared and burette reading was noted as volume of NaOH consumed. Acidity was calculated as:

$$\% \text{total acidity} = \frac{\text{Titre} \times \text{Normality of alkalinity} \times \text{eq. wt. acid} \times 100}{\text{Volume of sample taken} \times 1000}$$

4.4.4 Brix Percentage (Total Soluble Solids)

The determination of total sugar present in the test sample was carried by using refractometer or Brix method. Refractometer having different capacity were used according to expecting the results. Method essentially consists of following steps.

- i) The refractometer was adjust to zero using distilled water, the instrument was zeroed at 20°C
- ii) Temperature of test sample should be recorded.
- iii) Drops of sample was placed in refractometer and concentration was measured in terms of percentage
- iv) Mean of reading was calculated and accurate value was calculated according to ATAGO catalogue. (Calculation example is shown in Annex-3)

4.4.5 Alcohol Percentage

Alcohol percentage of alcoholic beverage was determined in followings two ways which consists of following steps.

a) Using Alcohol Meter

- i) The test sample was poured into clean beaker
- ii) The alcohol meter was dipped into sample
- iii) The readings were recorded in the percentage of alcohol presence in sample

b) Pycknometry Method

- i) 200 ml of sample with 25 ml D/W and 2/4 pieces of pumis stone were taken in 500 ml of distillation flask.
- ii) Distillation was carried out
- iii) Distillate was collected into 200 ml of measuring flask
- iv) Distillation should be continued up to 35 minute
- v) Flask cooled down in room temperature and distilled water was added and made volume up to 200 ml.

- vi) With the help of pycnometer (15/15°C), specific gravity was calculated and alcohol percentage was determined by V/V.

4.5 IDENTIFICATION OF MICROORGANISMS

Bacteria isolated on respective selective or different medium were identified by standard microbiological method following Bergey's Manual of Systematic Bacteriology (1986). The isolated bacteria were identified on the basis of colonial characteristics, morphological characteristics and biochemical properties.

4.5.1 Biochemical Tests for Identification of the Bacteria

Appropriate biochemical tests were done for identification of the bacteria. The isolated pure colonies were inoculated into different biochemical medium for the test as shown in table.

| Biochemical Medium | Tests |
|--|--|
| 1) Hugh and leifson media (O/F) | acid production in both either open or sealed tube |
| 2) 1% tetremethy 1 paraphenylene diamine | oxidase |
| 3) 3% hydrogen peroxide | catalase |
| 4) Plasma | coagulase |
| 5) Carbohydrate fermentation broth | mixed acid fermentation |
| 6) Peptone water | indole |
| 7) Simmon's citrate | utilization of citrate |
| 8) Triple Sugar Iron agar | H ₂ S production and gas production |
| 9) Urea agar | Urease |
| 10) Starch containing media | Starch hydrolysis |
| 11) Gelatin containing media | gelatin hydrolysis |
| 12) Blood agar | haemolysis |
| 13) MR-VP medium | MR and VP test |
| 14) growth in anaerobic agar | aerobic/anaerobic |
| 15) growth in high temperature | Thermophilic |
| 16) Antibiotic sensitivity test | sensitive/resistance |

Note: Composition and preparation of media used for isolation and identification of bacteria Gram's staining procedure.

4.6 QUALITY CONTROL FOR TESTS

Sterility was checked by incubating one or two uninoculated tubes and plates of the batch along with inoculated one. Batch of medium were discarded when inoculated tubes or plates showed the growth.

Control tests were also performed to confirm that the medium especially biochemical medium had been made up correctly. One tube of each batch of test medium was inoculated with a known culture of bacteria giving positive test and another tube with bacteria giving negatives tests.

4.7 MATERIALS

4.7.1 Equipment

1. Microscope - OLYMPUS-Japan 528918 MODEL CHD
2. Autoclave Atlas (Atlas surgical co. India)
3. Hot Air Oven - Memmert GmbH+COKG 8540 (Schwabach West Germany)
4. Incubator - Memmert GmbH+COKG 8540 (Schwabach West Germany)
5. Refrigerator - Phoenix BC- 152
6. Brix meter (0-32%)
7. Alcohol meter - Gay Jussac at 15 c Lemico, India
8. MF suction apparatus - Anand Medicals Pvt. Ltd., India
9. Membrane filter - Millipore type- HA
10. pH- meter - Hanna Instruments Ltd. Mauritius

4.7.2 Media Used

Culture media:

- | | |
|--------------------------------|----------------------|
| 1) Nutrient agar (NA) | HI- MEDIA LABORATORY |
| 2) Nutrient broth (NB) | HI- MEDIA LABORATORY |
| 3) Plate count agar (PCA) | HI- MEDIA LABORATORY |
| 4) Violet Red Bile agar (VRBA) | HI- MEDIA LABORATORY |
| 5) Potato Dextrose agar (PDA) | HI- MEDIA LABORATORY |
| 6) Mannitol Salt agar (MSA) | HI- MEDIA LABORATORY |
| 7) MacConkey agar (MA) | HI- MEDIA LABORATORY |
| 8) Cetramide agar | HI- MEDIA LABORATORY |

4.7.3 Biochemical Media

- 1) Hugh and Leifson's media (O/F) HI- MEDIA LABORATORY
- 2) Simmons citrate agar HI- MEDIA LABORATORY
- 3) Triple Sugar Iron agar (TSI) HI- MEDIA LABORATORY
- 4) Urea contain media HI- MEDIA LABORATORY
- 5) Carbohydrate fermentation media
- 6) Nitrate Reduction media
- 7) Indole
- 8) Methyl Red
- 9) Vogs Prausekar Test

4.7.4 Sensitivity Testing Media

1) Muller Hinton Agar (MHA) HI- MEDIA LABORATORY

4.7.5 Chemical Used

1) Staining Reagent

- | | |
|-------------------|--|
| 1) Crystal violet | Loba chemicals, India |
| 2) Iodine | Loba chemicals, India |
| 3) Alcohol | Benegal chemical and pharmaceutical, India |
| 4) Safranin | Loba chemical, India |

II) Test Reagent

- | | |
|---|-------------------------------------|
| 1) 3% hydrogen peroxide | Loba chemical, India |
| 2) Tetramethyl Paraphenylene Diamine Dihydrochloride | S.D. fine chemicals, India |
| 3) Potassium hydroxide | Central drugs house Pvt. Ltd. India |
| 4) Mercuric chloride | E. merck (India) limited |
| 5) Methyl red | Qualigens Fine Chemicals, India |
| 6) Ethyl alcohol | Qualigens Fine Chemicals, India |
| 7) Hydrochloric acid | Qualigens Fine Chemicals, India |

4.7.6 Antibiotic Disc Used

Antibiotic discs for sensitivity tests was from -Span Biotech. Pvt. Ltd. India.

CHAPTER-V

5. RESULTS

5.1 SURVEY RESULTS

Study was conducted by random sampling to cover the Kathmandu Valley. During survey retailer were asked about collection time of beverages from the manufacturer and average time taken for the consumption. At the same time, batch number and date of sampling (Annex-2) were noted for each sample.

Alcoholic beverages found abundantly in Kathmandu Valley. The storage condition of beverages was found to be very poor i.e. only certain numbers of beverages were in refrigerator. Beverages were collected from the shelves and jand, samples were collected in large vessel.

Beer was available in bottles while juicy and *raksi* were in plastic pouch, but *jand* was sold openly in a large container. During survey, sanitary conditions of the surrounding were also observed and were found to be very poor in most of the cases.

5.2 MICROBIOLOGICAL STUDY

5.2.1 Total Bacterial Count

During the study five of each different alcoholic beverages were analyzed. Microbial counts of different beverages from different sampling sites were carried out, according to standard plate count method and membrane filter technique. Membrane Filter Technique was applied to capture the micro-organisms presence in beverages using high volume of sample. During the study, beer sample was tested by membrane filter technique because beer was suspected to have low micro-organisms. By using this technique, total counts, coliform counts and yeast and mold count were carried out separately. The results are presented in table No. 1,2 respectively.

Table 1: Total Plate Count of different Alcoholic Beverages

| Sample No. | <i>Jand</i> | <i>Raksi</i> | Beer (MF-method) |
|-------------------|---------------------|---------------------|-------------------------|
| S ₁ | 0 | 0 | 128 |
| S ₂ | 5.1×10 ⁵ | 0 | 67 |
| S ₃ | 24×10 ⁵ | 0 | 123 |
| S ₄ | 127×10 ⁵ | 0 | 43 |
| S ₅ | 41×10 ⁸ | 0 | 23 |

The results are expressed in terms of Colony Forming Unit per ml (cfu/ml). While the total count of beer was done by -MF technique, expressed in cfu/100ml.

5.2.2 Coliform Count

All samples were analyzed for coliform group of bacteria. The coliform count was nil in almost all the samples i.e. there was absence of coliform in most of the alcoholic beverages samples. Similarly, coliform count was done by MF-technique in case of beer but there was absence of coliforms in all samples of beer.

5.2.3 Yeast and Mold Count

Yeast and mold were recorded in every sample of beverages. Comparatively, yeast were very common in almost all of samples, while molds were rare.

Table 2: Yeast and Mold Count of Different Alcoholic Beverages

| Sample No. | <i>Jand</i> | <i>Raksi</i> | Beer (MF-method) |
|-------------------|---------------------|---------------------|-------------------------|
| S ₁ | 0 | 0 | 134 |
| S ₂ | 21×10 ⁵ | 0 | 139 |
| S ₃ | 187×10 ⁵ | 0 | 135 |
| S ₄ | 32×10 ⁵ | 0 | 37 |
| S ₅ | 19×10 ⁸ | 0 | 39 |

The results express in cfu/ml, while total numbers of yeast and mold count of beer expressed in cfu/100.

5.3 BACTERIAL COUNTS OF DIFFERENT TYPES OF BEVERAGES

The results of total count, coliform and yeast and mold count were obtained by analyzing five samples of each three different alcoholic beverages.

5.4 MICRO-ORGANISMS ISOLATED FROM ALCOHOLIC BEVERAGES

The characterization of the isolated micro-organisms were mainly focussed on the bacteria, yeast and mold. The identification of bacteria and yeast were done by colony characteristics and biochemical tests where as the fungus was identified by their morphological characteristics of spore and mycelium. The colonial and biochemical characteristics of isolated bacteria and yeast and fungus were tabulated in table 4,5,6,7,8,9,10,11,12,13,14 and 15 respectively.

5.4.1 Morphological Characteristics of Isolated Micro-organisms

Table 3: Cultural characteristics of Gram Positive Cocci

| Cod e | Shape | Color | Margin | Consistenc y | Opacity | Elevatio n | Probable |
|--------------|--------------------|--------------|---------------|---------------------|----------------|-------------------|----------------------------|
| Ni | coccus in cluster | yellow | entire | Soft | translucent | raised | <i>Staphylococcus</i> sps. |
| Yc | coccus in cluster | yellow | entire | Soft | translucent | raised | <i>Staphylococcus</i> sps. |
| Nii | coccus in chain | cream white | entire | Soft | opaque | convex | <i>Streptococcus</i> sps. |
| Bii | coccus in tetroids | yellow | regular edge | Soft | opaque | convex | <i>Micrococcus</i> sps. |
| Wc | coccus | pale yellow | circular | Soft | opaque | convex | <i>Micrococcus</i> sps. |

Table 4 : Cultural characteristics of Gram Positive Rods

| Code | Shape | Spore | Color | Margin | Consistency | Opacit y | Elevation | Probable |
|-------------|-------------------------|--------------|--------------|-----------------|--------------------|-----------------|------------------|---------------------------|
| Bi | rods in chain | central | cream white | irregular | soft | opaque | dull | <i>Bacillus</i> sps. |
| Bc | rods in chain | central | white | round to spread | soft | opaque | dull | |
| Ba | rods in chain or single | central | cream white | undulate | soft | opaque | flat | <i>Bacillus</i> sps. |
| Be | rods in chain or single | central | cream white | undulate | soft | opaque | flat | <i>Bacillus</i> sps. |
| Bb | rods in chain | central | cream white | undulate | soft | opaque | flat | <i>Bacillus</i> sps. |
| Bd | rod | central | cream white | spreading | soft | opaque | flat | <i>Bacillus</i> sps. |
| Wd | straight long rod | no spore | white | entire | soft | opaque | convex | <i>Lactobacillus</i> sps. |

Table 5: Cultural characteristics of Gram Negative Rods

| Code | Shape | Spore | Color | Margin | Consistency | Opacity | Elevation | Probable |
|------|-----------|----------|-----------|-----------|-------------|---------|--------------|--------------------------|
| Gi | Short rod | no spore | greenish | round | soft | opaque | convex | <i>Pseudomonas</i> sps. |
| Wa | short rod | no spore | white | circular | soft | opaque | Raise convex | <i>Yersinia</i> sps. |
| Wb | short rod | no spore | Yellowish | irregular | soft | opaque | | <i>Enterobacter</i> sps. |

Table 6: Morphological Characters of Isolated Yeast

| Code | Characteristics feature | Probable Organism |
|------|--|---------------------|
| Ya | white to cream, butyrous colonies, budding cells | <i>Pichia</i> sps. |
| Yb | white to cream, butyrous colonies, budding cells | <i>Candida</i> sps. |
| Yc | white to cream, butyrous colonies, budding cells | <i>Pichia</i> sps. |
| Yd | pinkish colonies budding cell | <i>Candida</i> sps. |
| Ye | white, butyrous colonies, budding cells | <i>Candida</i> sps. |
| Yf | white, budding cells, circular colony | <i>Candida</i> sps. |
| Yg | white, budding cells, circular colony | <i>Pichia</i> sps. |
| Yh | white, budding cells, circular colony | <i>Pichia</i> sps. |
| Yi | white, budding cells, circular colony | <i>Pichia</i> sps. |
| Yj | white, budding cells, circular colony | <i>Pichia</i> sps. |

Table 7: Morphological Characters of Isolated Fungus

| Code | Characteristics feature | Probable |
|------|--|-----------------------|
| Jf | First white, partly immersed compact, partly effused-Backside black, mycelium are thin, highly branched transparency, stroma present | <i>Arthinium</i> sps. |

5.4.2 Biochemical Tests Performed for Identification of Bacteria

Table 8: Biochemical Tests for *Staphylococcus aureus*

| Code | Gram's | Shape | Catalase | Oxidase | O/F | Growth on MSA | Coagulase test | organism |
|------|--------|-------|----------|---------|-----|------------------|----------------|------------------------------|
| Yc | +ve | Cocci | +ve | -ve | F | Yellowish colony | +ve | <i>Staphylococcus aureus</i> |
| Ni | +ve | Cocci | +ve | -ve | F | Yellowish colony | +ve | <i>Staphylococcus aureus</i> |

O/F= oxidative or fermentative

Table 9: Biochemical Tests for Identification *Micrococcus* sps

| Code | Gram nature | Shape | Catalase | Oxidase | O/F | Citrate | NRT | Glucose fermentation | Mannose | Lactose | Organism |
|------|-------------|--------|----------|---------|-----|---------|-----|----------------------|---------|---------|--------------------|
| Bii | +ve | Coccus | +ve | O | +ve | -ve | +ve | -ve | -ve | -ve | <i>M.leutus</i> |
| Ni | +ve | Coccus | +ve | O | +ve | -ve | -ve | +ve | -ve | -ve | <i>M.kristinae</i> |

NRT= Nitrate Reduction Test

Table 10: Biochemical Tests for Identification of *Streptococcus* sps

| Code | Gram nature | Shape | Catalase | Oxidase | O/F | Hemolysis | Growth on K1 Streptococcal agar | Organism |
|------|-------------|--------|----------|---------|-----|---------------|---------------------------------|---------------------------|
| Nii | +ve | Coccus | -ve | -ve | F | Non hemolytic | +ve | <i>Streptococcus</i> sps. |

Table 11: Biochemical Tests for Identification of *Bacillus* sps

| Code | Gram nature | Catalase | VP | Citrate | Indole | Starch hydrolysis | Gelatin hydrolysis | NRT | Glu. Fermentation | Arabinose | Mannitol |
|------|-------------|----------|-----|---------|--------|-------------------|--------------------|-----|-------------------|-----------|----------|
| Ba | +ve | +ve | +ve | -ve | +ve | +ve | +ve | -ve | +ve | -ve | +ve |
| Bb | +ve | +ve | +ve | -ve | +ve | +ve | +ve | +ve | +ve | -ve | +ve |
| Bc | +ve | +ve | +ve | -ve | +ve | +ve | +ve | +ve | +ve | -ve | +ve |
| Bd | +ve | -ve | +ve | -ve | +ve | +ve | +ve | +ve | +ve | +ve | +ve |
| Be | +ve | +ve | +ve | -ve | +ve | +ve | +ve | -ve | +ve | -ve | +ve |
| Bi | +ve | +ve | +ve | -ve | +ve | +ve | +ve | +ve | +ve | -ve | +ve |

Ba, Be = *B. macerans*

Bb = *B. cereus*

Bc, Bi = *B. subtilis*

Bd = *B. larvae*

Table 12: Biochemical Tests For *Lactobacillus* sps.

| Code | Gram nature | Sucrose fermentation | Catalase | Oxidase | NRT | Starch hydrolysis | Mannose fermentation | Maltose | Organism |
|------|------------------|----------------------|----------|---------|-----|-------------------|----------------------|---------|---------------------------|
| Wd | +ve. No spore | +ve | -ve | +ve | -ve | +ve | +ve | -ve | <i>Lactobacillus</i> sps. |

Table 13: Bio-chemical Tests for Identification of Gram Negative Rods

| Code | Gram nature | Shape | Catalase | Oxidase | O/F | Citrate | Indole | Growth on mac conkey | Motility | MR | VP | TSI | Sensitivity |
|------|-------------|-----------|----------|---------|-----|---------|--------|----------------------|----------|-----|-----|-------|-------------------------|
| Gi | -ve | Short rod | -ve | -ve | O | -ve | -ve | NLF | Motile | ND | ND | ND | ND |
| Wa | -ve | Short rod | -ve | -ve | ND | -ve | ND | NLF | ND | +ve | -ve | Alk/A | Sensitive to penicillin |
| Wb | -ve | Short rod | -ve | -ve | ND | | -ve | NFL | ND | -ve | -ve | A/A | ND |

Gi: *Pseudomonas* sps. WA= *Yersinia* sps. Wb=*Enterobacter* sps.

ND= Not Done NLF = Non Lactose Fermenter

Table 14: Bio-chemical Tests for Identification of Yeast

| code | Glu. Fermentation | Lactose | Maltose | Galactose | rafinose | Sucrose | urease | NRT | citrate | Strch hydrolysis | Organism |
|------|-------------------|---------|---------|-----------|----------|---------|--------|-----|---------|------------------|----------|
| Ya | +ve | +ve | -ve | +ve | +ve | -ve | -ve | -ve | +ve | +ve | |
| Yb | +ve | +ve | -ve | +ve | -ve | +ve | -ve | -ve | +ve | +ve | |
| Yc | +ve | +ve | +ve | +ve | -ve | +ve (w) | -ve | -ve | +ve | +ve | |
| Yd | +ve | +ve | +ve | +ve | +ve (w) | +ve | +ve | +ve | +ve | -ve | |
| Ye | +ve (w) | -ve | +ve | -ve | -ve | +ve | -ve | -ve | +ve | +ve (g) | |
| Yf | +ve | +ve | +ve | -ve | -ve | +ve | -ve | +ve | +ve | +ve | |
| Yg | +ve | +ve | ND | +ve | ND | +ve | ND | +ve | +ve | +ve | |
| Yh | +ve | ND | ND | ND | ND | +ve | ND | +ve | +ve | +ve | |
| Yi | +ve | ND | ND | ND | ND | +ve | ND | +ve | +ve | +ve | |
| Yj | +ve | ND | ND | ND | ND | +ve | ND | +ve | +ve | +ve | |

(w)= weak (g) = gas ND= Not Done NRT = Nitrate Reduction Test

5.5 PARAMETERS

This topic includes the analysis results of different chemical parameters such as: pH, Acidity, Brix and Alcohol percentage of different alcoholic beverages. These parameters are important for the quality of the products. Analyzed parameters as shown in table No. 16, 17 and 18 respectively.

Table 15: pH (Hydrogen Ion Concentration) of Different Alcoholic Beverages

| Sample No. | Beer | <i>Jand</i> | <i>Raksi</i> |
|----------------|------|-------------|--------------|
| S ₁ | 4.53 | 4.02 | 3.9 |
| S ₂ | 4.56 | 4.02 | 4.01 |
| S ₃ | 4.56 | 4.02 | 4.02 |
| S ₄ | 4.56 | 4.02 | 3.82 |
| S ₅ | 4.56 | 4.02 | 3.98 |
| Mean | 4.55 | 4.02 | 3.98 |

Table 16: Acidity (as Citric acids or Acetic Acids or Tartaric Acid Percentages)

| Sample No. | Beer (Tartaric Acid) | <i>Jand</i> (Acetic Acid) | <i>Raksi</i> (Tartaric Acid) |
|----------------|-------------------------|---------------------------|------------------------------|
| S ₁ | 0.136 | 0.312 | 0.0151 |
| S ₂ | 0.151 | 0.270 | 0.0151 |
| S ₃ | 0.158 | 0.282 | 0.0151 |
| S ₄ | 0.151 | 0.270 | 0.0151 |
| S ₅ | 0.158 | 0.270 | 0.0151 |
| Mean | 0.151 | 0.280 | 0.0151 |

Table 17: "Brix (Total soluble Solids as Percentages) of different Alcoholic Beverages

| Sample No. | Beer | <i>Jand</i> | <i>Raksi</i> |
|----------------|-------|-------------|--------------|
| S ₁ | 5.04 | 3.35 | 6.81 |
| S ₂ | 5.14 | 3.35 | 6.81 |
| S ₃ | 5.14 | 3.15 | 6.81 |
| S ₄ | 5.33 | 3.35 | 6.81 |
| S ₅ | 5.33 | 3.35 | 6.81 |
| Mean | 5.196 | 3.31 | 6.81 |

Table 18: Alcohol Percentage of Alcoholic Beverages

| Sample No. | Beer (v/v) | <i>Jand</i> (v/w) | <i>Raksi</i> (v/v) |
|----------------|------------|-------------------|--------------------|
| S ₁ | 5.15 | 5 | 22 |
| S ₂ | 5.15 | 5 | 23 |
| S ₃ | 5.15 | 4 | 23 |
| S ₄ | 5.15 | 5 | 23 |
| S ₅ | 5.15 | 4 | 23 |
| Mean | 5.15 | 4.6 | 22.8 |

CHAPTER- SIX

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

This work was designed to investigate the microbial as well as chemical parameters of alcoholic beverages. Demand for varieties of alcoholic beverages in hotels, restaurant, cold store is increasing day by day. Bacteriological and chemicals safety of alcoholic beverages were of comparable hygienic quality. The work also targeted to study external contamination of beverages. Similarly determination of chemical parameters, such as pH, 'Acidity, Brix and Alcohol percentage. The samples were collected randomly from the different retail shops i.e. five of each different beverages from the different sampling sites coverage Kathmandu valley.

Bacteriological study was carried out by Pour Plate Technique and MF-Technique. In this work microbiological study covered- Total Bacterial Count, Coliform Count and Yeast and Mold Count. Total plate count method is generally accepted. Similarly, coliform group of bacteria is important quality control of alcoholic beverages as well as possible contamination of coliform in foods is considered to be an indicator of degree in sanitary practices during the production, processing, and handling the products. Yeast and mold count is also important to keep the qualities of food products, because yeast and mold are also possible sources for the spoiling the products.

Microbiological Parameters

Our results showed that *jand* had higher in total plate count and in yeast & mold count. In the alcoholic beverages the highest count was found in *jand* i.e. 4×10^8 cfu/ml. Total count of beers was ranging from 23 to 128 cfu/100ml.

Amongst alcoholic beverages, highest count (for yeast and mold) was noted in *jand* i.e. 19×10^8 , While nil in *raksi*. But low numbers of yeast and mold count

was in beer ranges i.e. 37 to 139 cfu/100ml. Coliform counts in alcoholic beverages were almost nil.

There are many possibilities of contamination due to handling during processing of food. Food handler has great role to play in food hygiene and from a public health aspect, this source of contamination has considerable attention.

The study includes some alcoholic beverages like beer, *raksi* (Nepali local brand) and *jand* available in Newari community of Kathmandu valley.

Most of beers found in Nepali market are manufactured in collaboration with International companies. Hence, they must meet the international standards as well as Nepali standard. Microbial analysis was carried out by membrane filter technique (MF- Technique). This technique was applied to capture the organisms present in sample using high volume i.e. 100 ml of sample. The analysis showed that all of the samples were contaminated.

In beer, total bacterial count was ranging from 23 cfu/100ml to 128 cfu/100ml as shown in Fig 2 i.e. the samples No. S₁ (128 cfu/100 ml) and S₃ (123 cfu/100 ml) were highly contaminated. The bacterial count showed that, there were two different types of predominant bacteria. One was with white large colony, while another was yellowish white colonies. Biochemical and morphological study confirmed that the isolated bacteria were *Bacillus subtilis*, and *Staphylococcus aureus*. The isolates were pathogenic as well as brings undesirable changes within the products. This indicates the microbial quality of beer was low and threatens to consumer. *Staphylococcus aureus* was found 2 cfu/100ml and *Bacillus* sps. were predominant in all samples.

While another alcoholic sample was Nepali local alcoholic beverages i.e. "*raksi*", contains 23% of alcohol distributed in plastic pouch. During the survey, it was found that such *raksi* was highly consumable in low economic society. Bacteria as well as yeast and mold counts were not found in *raksi* Fig. 3 and 4 showing the count of yeast and mold.

The microbial and chemical analysis of samples were done as previous sample. During the microbial examination, the product was found free from any micro-

organisms i.e. zero total count, coliforms, as well as yeast and mold counts. Results indicate that the alcoholic concentration of the product, because alcohol acts as a good disinfectant. On the other hand, alcohol is a coagulant and denature cell proteins which is most germicidal in concentration between 70 and 95%. Though *raksi* contains only 23% of alcohol but it was possible that the contact period between micro-organism and alcohol may be long, hence chances of zero microbial count in *raksi* is more.

Jand is Nepal traditional alcoholic beverage in which microbial load was very high. This indicates that the quality of product is very low. The total plate count was ranging from 5.1×10^5 cfu/ml to 41×10^8 cfu/ml i.e. Fig. 2 showed the highest count of bacteria in sample No. S₅ and different types of bacteria were found i.e. yellowish white colony was 11×10^4 cfu/ml in almost all samples while, another white colony were observed in the range of 35×10^4 cfu/ml to 127×10^5 cfu/ml. The morphological and biochemical test confirmed the isolates were *Yersinia* sps. , *Micrococcus* sps. , similarly, yeast and mold count was shown in Fig. 4. Among the five samples, sample No. S₅ showed the highest count.

Y. pseudotuberculosis causes acute mesenteric Tymphadeniti (inflammation of the lymph glands in the membranes that support the intestine). The condition can cause serve low or abdominal pain. Occasionally, *Y. pseudotuberculosis* invades the blood causing septicemia is usually fatal. Most *Y. pseudotuberculosis* infections have been reported from Europe. The incidence of the disease in tropical countries is not known. (Cheesbrough, 1984).

Presence of *Streptococcus* sps. indicates the product has been contaminated. The *Streptococci* sps. are widely distributed being found in air, water, \soil and in various food products. *Micrococcus* sps. are found in nature, like: soil, water and in many foods. As we have already discussed importance source of *Bacillus* sps. is soil. Among *Bacillus* sps. *B subtilis* and *Bacillus cereus* are important for spoilage of many foods. Hence, presence of these bacteria in *jand* can be due to contaminated with water used for production of *jand* as well as use of unsterile

utensils and unhygienic handling. The results showed that *Bacillus* sps. was found in almost all samples of alcoholic beverages, except *raksi*.

Lactobacillus sps. was also isolated which are widely distributed among plants and in dairy products. Similarly, *Enterobacter* sps. which is found on plant surface and in soils more often than in intestinal contents.

Source of Micro-organisms

The microbial analysis of different alcoholic beverages showed much diverse type of micro-organisms. The microbial flora of beverages consists of the micro-organisms associated with raw material, those acquired during handling and processing and those surviving any preservation, treatment and storage. Since, micro-organisms do not arise by spontaneous generation they must contaminate the food at some stage of production, handling, processing, storage and distribution. Most foods are subjected to contamination by many potential sources of micro-organisms. The potential sources of contamination are soil, water, air, processing equipment, ingredients to product and packaging material. Water is potential source of microbial contamination of alcoholic beverages. Water is used during production and processing. The effect of air has on the micro flora of food depends upon the level of contamination of air and the time of contact of air with food. During the course of the industrial revolution, machines were developed. Hence, there is less contact with food by humans and more contact by machine and equipment. Processing equipment is one of the major source of contamination. The types of micro-organisms in an ingredient or product are often more important than the total numbers that are present. Organisms that cause spoilage or are potential health hazards are the important ones to consider in ingredients or final products. Packaging is a potential source of contamination. There are many studies concerning the effect of different types of packaging material on the shelf-life of alcoholic beverages.

Packages serve as a protective covering to limit or prevent microbial contamination; however they do not prevent microbial growth. It is important that preparation of the food for packaging is designed to limit or prevent

microbial contamination prior to packaging. Also, the package must be durable enough to maintain its integrity during storage and distribution.

All foods and food product undergo spoilage by the action of micro-organisms which gain entry into the products during production, processing and packaging. Some of the organism may be pathogenic. These are toxin producing types giving rise to food borne infection.

Yeast and mold counts indicates the presence of spoilage and harmful types of yeast and mold. In either case, a high level of mold indicates poor processing technique. The presence of mold may indicate the possible presence of mycotoxins which can be a health hazard. According Food and Drug Administration (1973) mold defects action apart in fruits juices is average of 10%.

Contaminated foods were not always deleterious to human health, such foods are unacceptable from an aesthetic point of view and are considered unfit for consumption.

Parameters

Physical parameters are also important for keeping accurate quality of alcoholic beverage. The analyzed physical parameters were: Temperature, pH, Acidity, Brix (total soluble solids) and Alcohol percentage. These parameters should be accurate otherwise these may bring undesirable changes within the product e.g. temperature, ie. (Storage temperature) was known by measuring the temperature and can be gussed, the conditions of the alcoholic beverages. Similarly, pH of alcoholic beverages should be accurate because this may favour the growth of different types of micro-organisms as well as decrease or increase in pH lead to unacceptable of alcoholic beverages. Another parameter was Brix percentage, which also determine the quality of alcoholic beverages and again high percentage of sugar may favour the growth of different types of micro-organisms. Alcohol percentage, should be measured because classification of beer and wine depends upon alcohol percentage therefore, there is statutory

regulation in case of alcohol percentage which is also important parameter of alcoholic beverages.

Together with the food reports of the food additives and chemical parameters and limits have been recommended, many of which have been incorporated into statutory regulations.

Temperature:

This is extrinsic parameters of foods. This property of the storage environment that affect the both foods and their micro-organisms. Micro-organisms grow over a very wide range of temperature. Therefore, it would be better to consider that the temperature growth ranges for organisms of importance in foods as aid in selecting the proper temperature for the storage of different types of foods.

The recorded temperature showed in Annex-1. The recorded temperature of analyzed beverages lie within the range of 14⁰ C to 23.5⁰ C.

pH:

The pH of the alcoholic beverages were measured by pH-meter. Standard the pH of beer should lies between 3.0 to 4.8 (NS:44/2044). This indicates that the pH of the beer was within the range. In case of *jand*, the pH depends upon fermentation days but results showed that the pH value was more or less same in all samples i.e. 4.06. In *raksi*, the pH was ranging from 3.9 to 4.02, the mean value was 3.89. This indicates that *raksi* was acidic in nature. This may be due to long fermentation period. The graph showing pH of different alcoholic beverages (Fig. 6).

The pH of a product can be readily determined with a pH meter, but this value along may not be sufficient for predicting microbial responses. Microorganism survive during storage, heating, drying and other forms of processing. The initial pH may vary because of competitive flora or growth of the organisms.

Every micro-organism has a minimal, a maximal and an optimal pH for growth, conversely, the initial pH may be restrictive but the growth of a limited number of micro-organisms may later the pH to more favorable ranges for the growth of many other micro-organisms. (Frazier and Westhoff, 1978)

Hence, pH is important parameter for keeping quality of beverages microbiological as well as chemicals. It is of importance as a measure of the active acidity which influence the flavour or palatability of a product and affects the processing requirements. The overall range of pH for the common fruit juices is 2 to 5 and the most common range is between 3 to 4. Variation in pH even as small as 0.15 to 0.20 may often be significant. The pH values of different alcoholic beverages is shown in Fig. 5 and 6 respectively whereas carbonated drinks was acidic among the non-alcoholic beverages and *raksi* was acidic in nature amongst alcoholic beverages.

Acidity:

Each alcoholic beverage has their own titrable acidity expressed in different acid e.g. fruit juices measures as citric acids, beer as lactic and acetic acids. Acidity has relation to pH. According to the value of pH, acidity can be altered e.g. fermented products have a low pH because of developed acidity, it means that increase in acidity lowers the pH.

Acidity of beer, *jand* and *raksi* was measured as acetic acid and tartaric acid respectively. In a *raksi* acidity was measured as atartaric acid which is present partly as the free acid, partly as cream of tarter with a little calcium tartarate. The total tartaric acid present in *raksi* may vary from 0.07 - 0.5% (Pearson, 1976). Our result showed (Table-17) that acidity of *raksi* was 0.0151. The mean value was also 0.0151%. The acidity of *raksi* was comparatively low. The acidity of beer was measured as tartaric acid, while acetic acid represent the volatile acidity. The acidity of beer examined were ranging from 0.136 to 0.158%, while mean value was 0.151 and acidity of *jand* was 0.28% (The acidity of alcoholic beverages is shown in Fig. 8).

Brix:

The total soluble solids (Brix percentage) of beverages were measured using refractometer. The amount of sugar percentage recorded during the study was more or less same to the recommended value but different condition of storage, processing, handling etc. may be causes of change in Brix value.

The total soluble solids play an important role in determining the quality of alcoholic beverages because sugar enhances the growth of different types of microflora, chiefly yeast and bacteria. On the other hand, sugar defines the taste of beverage, hence it should be accurate. In case of alcoholic beverage sugar percentage is also important. It also helps in keeping the quality of alcohol. In alcoholic beverages, Brix reading is defined in separate way than in soft drinks.

Alcohol Percentage:

Alcohol percentage of alcoholic beverage is an important parameter. In this study we tried to determine the alcoholic percentage of beverages in two different ways i.e. volume/weight and volume/volume using two different methods. Among the alcoholic beverage we studied alcoholic percent of beer, *jand* and local *raksi*.

The average value of alcohol percentage of beer was 5.15% v/v. Nepal Standard recommended the percentage of alcohol of beer should lie 2.0 to 6.5% v/v (NS: 23/2040), hence comparatively, study suggested that the alcohol percentage of tested beer was within the range.

In case of *jand*, the alcohol percentage was ranging from 4 to 5% v/w. *Jand* is Nepali type of beer i.e. traditional alcoholic beverages and alcoholic percentage was also lies within the range of beer. There was no defined standard limitation of alcohol percentage of *jand*. Next, alcoholic beverages in Nepali local *raksi* which showed the alcoholic percentage ranging from 22 to 23% v/v (the alcohol percentage of alcoholic beverages is shown in Fig. 11).

The experiment was conducted according to the international system of expressing alcoholic strength should be as the percentage of alcohol by volume at 20⁰C (Savage, 1973).

In alcoholic beverages, the percentage of alcohol is very useful to check the microbial growth to some extent because the alcohol acts as good disinfecting and long time of contact of micro-organisms with the alcohol may be the reason for low microbial count in *raksi* and *jand*. Alcohol is coagulant and denaturizes cell proteins. But alcoholic content of beer is not great enough to prevent their spoilage by micro-organisms. Hence, alcohol percentage is an important chemical parameter which also help in determining microbiological parameters.

6.2 CONCLUSION

The tested alcoholic beverages showed the microbial load contaminated but physiological parameters met the standard.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATION

7.1 SUMMARY

Alcoholic beverages from the retail shop of Kathmandu city marketed were bacteriological and chemically investigated to study its quality. During the bacteriological study, Pour Plate Technique and Membrane Filter Technique were applied, and for chemical analysis different standard (AOAC) methods were applied. The study was supported by the surveillance conducted randomly in Kathmandu valley.

The study analyzed microbial and chemical parameters separately. In case of microbiological tests- Total plate Count, and Yeast Mold counts were performed. While in chemical parameter; Temperature, pH, Acidity, Brix (total soluble solids) and Alcohol percentage were analyzed.

Bacteriological study showed that *Jand* were highly contaminated i.e. these two different beverages showed highest total plate count 1.48×10^7 cfu/ml and 41×10^8 cfu/ml respectively. In *Jand*, various types of bacteria were isolated i.e. *Yersinia* sps., *Enterobacter* sps., *Streptococcus* sps. and *Bacillus* sps. In case of yeast *Pichia* sps., *Candida* sps., were predominant.

In *raksi*, the total count was almost all nil. The coliforms count were totally nil in case of all of different samples of *raksi*. The microbial profile of alcoholic beverages showed that all most all of bacteria were natural and saprophytic bacterial flora. *Staphylococcus* group of bacteria may be contaminants from air and human source.

The chemical parameters: pH, Acidity, Brix and Alcohol percentage were within limits i.e the analyzed value tally the recommended value or required value. Nepal standard recommended the pH value of beer is within 3.0 to 4.8 but observed value was 4.55, it means that the pH values were within the

desirable range. Similarly, the pH of *Jand* and *raksi* were 4.02 and 3.93 respectively.

Acidity of alcoholic beverages was calculated as their titrable acidity in percentage tartaric acid. The average observed value of acidity of beer, *jand*, and *raksi* were 0.151, 0.28 and 0.0151 respectively. Usually alcoholic beverages contain very little volatile acidity except those contributed by aliphatic fatty acids which do not exceed a few milli equivalents. Volatile acidity at best serve as quality index.

Total soluble solids of respective alcoholic beverages measured as Brix reading by refractometer i.e. 5.196, 3.31 and 6.81 of beer, *jand* and *raksi* respectively.

Amongst the alcoholic beverages beer, *jand* (Nepali traditional alcoholic beverages) and Nepali local *raksi* contained 5.15, 4.6 and 22.8 alcohol percentage in averages respectively.

Hence it could be concluded that the different alcoholic beverages were microbiologically highly contaminated. During the study, the physical parameters of alcoholic beverages were also studied but there were no any physical changes recorded in any alcoholic beverages.

7.2 RECOMMENDATIONS

Since, micro-organisms do not arise by spontaneous generation they must contaminate the food at some stages of productions handling, processing, storage and distribution. Most foods are subjected to many potential source of contamination, hence to avoid any types of microbiological contamination followings are the precaution to be undertaken to improve the quality of alcoholic beverages.

- The ingredients of the alcoholic beverages should be purified i.e. free from chemicals and microbial contamination.

- Avoid external contamination during production, processing, packaging, storage and distribution.
- Bottles and closures are possible source of contamination hence these should be sterile.
- Food handlers have great role to play if food hygiene and from a public health, this sources of contamination has consideration attention.
- The microbial contamination may alter chemical parameters hence, microbial parameters should be properly monitored.
- The temperature of alcoholic beverages actually deals about storage temperature hence, temperature should be maintained while storage and distribution.
- Another important parameter is pH because alcoholic drinks have their own pH. This parameter defined the taste of alcoholic beverages as well favor the growth of different types of organism hence, it should be checked and maintained properly.
- Acidity of alcoholic beverages should also be maintained because alcoholic beverages have their own titrable acidity and alter in pH may bring change in acidity.
- In case of alcoholic beverages sugar percentage indicate remaining of sugar after all total fermentation. Hence, it should be measured to define the quality of alcoholic beverages.
- Alcoholic percentage of alcoholic beverages should be measured because on the basis of the alcohol percentage alcoholic beverages were classified e.g. beer and wine. Hence, it should be in accurate range for keeping the quality.
- There should be the framework for quality control of food, working under the government or under municipality. The present food act in Nepal should be either modified or amended

to cover broad aspects of public health through food safety. The present food act does not cover all the foods of daily use. It should be amended accordingly. In Nepal implementation of food act is weak and it should be made more practical and effective.

- There should be surveillance system for quality and quantity assurance from government.

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APPENDIX-I

| | SAMPLING SITE | SAMPLING DATE | TEMPERATURE (° C) |
|-----------------|----------------|---------------|-------------------|
| a) Beer | Naya Baneshwor | 053/09/16 | 14 |
| | Kupondol | 053/9/23 | 16 |
| | Kirtipur | 053/10/1 | 16 |
| | Bag Bazar | 053/10/7 | 16 |
| | Bhaktapur | 053/10/15 | 17 |
| b) <i>Raksi</i> | Naya Baneshwor | 053/9/4 | 17 |
| | Koteshwor | 053/9/7 | 17 |
| | Kalanki Chowk | „ | 17 |
| | Kupondol | „ | 17 |
| | Kirtipur | „ | 17 |
| c) <i>Jand</i> | Kirtipur | 053/9/26 | 16 |
| | Bag Bazar | 053/9/28 | 16 |
| | Bhaktapur | 053/9/29 | 16 |
| | Kalanki chowk | 053/10/1 | 16 |
| | Baneshwor | 053/10/1 | 16 |

APPENDIX-II

I. COMPOSITION AND PRERARATION OF MEDIA USED IN ISOLATION AND IDENTIFICATION OF BACTERIA

I. Culture Media:

1. Blood Agar:

Blood agar is used for isolationand cultivation of fastidious organism. Blood agar was used to study β - haemolytic activity of *Streptococcus* and *Staphylococcus aureus*.

Composition : Nutrient agar +5% blood.

Preparation:

The medium was prepared by adding 5% sterile blood to sterile nutrient agar that had been cooled down to 50⁰ c. It was poured into sterile petri plates aseptically.

2. Cetrimide Agar:

Cetrimide agar is a selective plate medium used occasionally to isolate *Pseudomonas* sps. from mixed bacterial flora. It inhibits the growth of bacteria such as *Staphylococcus aureus* and coliform.

| Composition | Grams/lt |
|------------------------------|----------|
| Peptone 190 | 20.0 |
| Potassium sulphate | 10.0 |
| Magnesium chloride | 14.0 |
| Cetrimethy lammonium bromide | 0.30 |
| Agar | 15.0 |

Preparation:

The medium used at a concentration of 4.67 grms in every 100ml of D/W and autoclaved and cooled down to 50-55⁰ C and poured to sterile plates.

3. MacConkey agar:

MacConkey agar used for detection of enteric organism including *Enterococci* and also of the gram positive cocci. MacConkey agar was used for detecting lactose fermenting capacity of isolated bacteria.

| Composition | Grms/Lt. |
|---------------------------------|----------|
| Peptone | 20.0 |
| Lactose | 10.0 |
| bile salts | 5.0 |
| sodium chloride | 5.0 |
| neutral red | 0.075 |
| Agar | 12.0 |
| final pH (at 25 ⁰ C) | 7.4±0.2 |

Preparation:

52 grams of media was dissolved in 1000ml of D/W heated to boil to dissolve it completely. It was sterilized by autoclaving at 15 lbs pressure for 15 minutes.

4. Mannitol Salt Agar

Mannitol salt agar is a selective medium for isolation of pathogenic *Staphylococcus* sps.

| Composition | Grms/Lt. |
|------------------|----------|
| beef extracts | 1.0 |
| Protease peptone | 10.0 |

| | |
|---------------------------------|---------|
| Sodium chloride | 75.0 |
| D-mannitol | 10.0 |
| Phenol red | 0.025 |
| Agar | 15.0 |
| final pH (at 25 ⁰ C) | 7.4±0.2 |

Preparation:

Ingredients were suspended in 1000ml D/W and boiled to dissolved the medium completely. It was sterilized at 15 lbs pressure 120⁰ c for 15 minutes.

5. Nutrient Agar

Nutrient agar is a general purpose media recommended for the vultivation of micro-organism which are non fastidious.

| | |
|---------------------------------|----------|
| Composition | Grms/Lt. |
| Peptone | 5.0 |
| Sodium Chloride | 5.0 |
| beef extracts | 1.5 |
| yeast extracts | 1.5 |
| Agar | 15.0 |
| final pH (at 25 ⁰ C) | 7.4±0.2 |

Preparation:

28 grams of the medium was suspended in 1000ml D/W. The medium was boiled to dissolved it completely and sterilized at 15 lbs pressure (121⁰c) for 15 minutes. Then cooled down to 50⁰ C and poured aseptically into sterile petriplates.

6. Nutrient Broth

Nutrient broth is used for general or routine cultivation of non fastidious organism.

| | |
|-------------|----------|
| Composition | Grms/Lt. |
| Peptone | 5.0 |

| | |
|---------------------------------|---------|
| sodium chloride | 5.0 |
| beef extracts | 1.5 |
| yeast extracts | 1.5 |
| final pH (at 25 ⁰ C) | 7.4±0.2 |

Preparation:

13 grams was suspended in 1000ml D/W and boiled to dissolved it completely. It was sterilized by autoclaved at 15 lbs pressure 121⁰ C for 15 minutes.

7. Plate Count agar

Plate count agar is used for the enumeration of abcteria in water, waste water dairy products and foods. Plate count agar is equipment to the medium recommended by APHA for plate count of micro-organism in milk and other dairy products.

| | |
|---------------------------------|----------|
| Composition | Grms/Lt. |
| tryptone | 5.0 |
| Yeast extracts | 2.5 |
| dextrose | 1.0 |
| agar | 9.0 |
| final pH (at 25 ⁰ C) | 7.4±0.2 |

Preparation

17.5 grams of the media was suspended in 1000ml D/W and boiled to dissolved it completely. The media was sterilized by autoclaving at 15 lbs pressure 121⁰ C for 15 munites. It was cooled to 50-55⁰ C and poured aseptically into sterile petriplates containing diluted samples.

8. Potato Dextrose Agar

Potato dextrose agar is used for isolation and cultivation of yeast and mold.

| | |
|-------------|----------|
| Composition | Grms/Lt. |
|-------------|----------|

| | |
|----------------|--------|
| Potato(peeled) | 200.0 |
| dextrose | 20.0 |
| agar | 15.0 |
| D/W | 1000.0 |

Preparation:

Peel of skin of the potato, cut into small pieces and boil (200g) in 500 ml water, till they are easily penetrated by the a glass rod. Filter through cheese cloth. And Dissolved agar in water and bring upto required volume by the addition of water.

9. Violet Red Bile Agar

Violet red bile agar is a selective medium for the detection and enumeration of coliform organism.

| Composition | Grms/Lt. |
|---------------------------------|----------|
| Yeaswt extract | 3.0 |
| peptone | 7.0 |
| bile salts No. 3 | 1.5 |
| lactose | 10.0 |
| sodium chloride | 5.0 |
| agar | 15.0 |
| neutral red | 0.03 |
| crystal violet | 0.002 |
| final pH (at 25 ⁰ C) | 7.4±0.2 |

Preparation

42.5 grams was suspended in 1000ml sterile D/W and heated to boil to dissolve the medium completely. The medium was not autoclaved. It was cooled to 45⁰ c and poured into sterile plates containing the inoculum.

II) BIOCHEMICAL MEDIA, BIOCHEMICAL TESTS AND ITS RESPECTIVE REAGENTS

1) Carbohydrate Utilization Test

Carbohydrate utilization tests are to determine the ability of an organism to degrade a specific carbohydrate incorporated in a basal medium producing acid or acid with visible gas.

| Composition | Grms/Lt. |
|--------------------------|----------|
| Peptone | 10.0 |
| beef extracts (optional) | 1.0 |
| sodium chloride | 5.0 |
| Phenol red | 0.018 |
| D/W | 1000ml |
| pH | 7.4 |

Preparation

The amount as indicated above was weight out accurately and boil to dissolve completely. 5ml of the medium was dispensed in test tube containing inverted Durham's tube and autoclaved at 15 lbs pressure (121⁰ C) for 15 minutes. Sterile stock solution of desired carbohydrate was added aseptically to each tube to give or final concentration of 1% (w/v).

Procedure

Test organism was inoculated into the medium and inoculated at 37⁰ C for 24 hrs. Production of acid (A) and gas (G) was observed.

2) Catalase Test

Catalase test is done the presence of enzyme catalase. The enzyme catalase splits hydrogen peroxides water and oxygen.

Reagents: 3% hydrogen peroxide

| Composition | Grms/Lt. |
|--|----------|
| Concentrated H ₂ O ₂ | 3ml |
| D/W | 97 ml |

Procedure

3ml of 3% hydrogen peroxide was taken in a test tube and colony of bacteria to be tested was picked up from nutrient agar with the help of glass rod and inserted into the tube containing reagent the production of gas bubbles immediately indicates positive catalase test.

3) Citrate Utilization Test

Citrate utilization test is performed to determine if an organism is capable of utilizing citrate as to solve source of carbon for metabolism with resulting alkalinity.

Medium :Simmon's citrate agar

| Composition | Grms/Lt. |
|------------------------|----------|
| Monoammonium phosphate | 1.0 |
| Dipotassium phosphate | 1.0 |
| Sodium chloride | 5.0 |
| Sodium citrate | 2.0 |
| Magnesium sulphate | 0.2 |
| Bromothymol blue | 0.08 |
| Agar | 15.0 |

final pH (at 25⁰C)

7.4±0.2

Preparation:

24.2 grams was suspended in 1000ml D/W. It was heated to boil to dissolve the medium completely. It was distributed in tubes and sterilized by autoclaving at 15 lbs pressure (121⁰C) for 15 mins. The medium in tube were solidified in started position.

Procedure:

The slant was streaked with test organism and incubated at 37⁰ c for 48 hrs. Growth of organism with an intense blue color on slant is the indicative of positive test, No growth with no change in color (green) is the negative test.

4) Coagulase Test:

The ability of an organism to clot plasma by the action of *enzyme* coagulase is tested by coagulase test. Thin test is used to differentiated *Staphylococcus aureus* which usually produces the enzyme coagulase from *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* which do not produce coagulase.

Procedure:

A drop of physiological saline was put on each of a slide. A colony of test organism from nutrient agar was emulsified in each of the drops to make tow thick suspension. A drop of plasma was added to one of the suspension and mixed gently. Clumping of organism within the 10 seconds is positive test.

5) Gelatin Hydrolysis Test.

Gelatin hydrolyzing capacity of bacteria is tested by gelatin hydrolysis test. Gelatin hydrolysis indicates the presence of enzyme gelatinase.

Composition: Nutrient agar and 1% gelatin

Preparation:

One gram gelatin was added to 100ml nutrient agar and boiled to mix well. It was sterilized by autoclaving at 121⁰C for 15 mins. It was poured on sterile petriplates and allowed to solidify.

Procedure:

Test organism was spot inoculated onto the solid gelatin agar medium and incubated at 37⁰C for 24 hrs. The plate was flooded with mercuric chloride solution, allowed it to stand for 5-10 minutes. A clear zone observed the growth in presence of mecuric chloride solution demonstrate hydrolysis of gelatin.

6) Hydrogen Sulfide Test (Triple Sugar Iron agar)

The test is done to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium, with or without the production of gas, along with determination of possible hydrogen sulfide production.

| Composition | Grms/Lt. |
|---------------------------------|----------|
| Peptone | 10.0 |
| tryptone | 10.0 |
| Yeast extract | 3.0 |
| beef extract | 3.0 |
| lactose | 10.10.00 |
| saccharose | 1.0 |
| dextrose | 0.2 |
| ferrous sulphate | 5.0 |
| sodium chloride | 0.3 |
| sodium thiosulphate | 0.024 |
| phenol red | 12.0 |
| agar | 12.0 |
| final pH (at 25 ⁰ C) | 7.4±0.2 |

Preparation

6.5 grams was suspended in 100 ml of D/W. It was distributed in tubes and sterilized by autoclaving at 15 lbs pressure (121⁰ C) for 15 minutes. The medium was allowed to set in slanted form with a butt about 1 inch long.

Procedure

The test organism was stabbed in the butt and streaked on the slant the tubes were incubated at 37⁰ for 24 hrs. Black coloration of butt, slant and gas formation was also noted and recorded as alkali/alkali, alkali/acid, acid/acid for the growth of a fermenters, glucose fermenters and all sugar fermenters.

Indole Test:

Indole test is done to determine the ability of an organism to split -indole from tryptophan molecule.

Medium-peptone water

| Composition | Grms/Lt. |
|-------------------------------|----------|
| Peptone containing tryptophan | 20.0 |
| sodium chloride | 5.0gm |
| D/W | 1000.0ml |

Procedure:

15 grams was dissolved in 1000 ml of D/W. It was boiled to dissolved completely, it was then dispensed in test tube and sterilized at 15 lbs pressure 121⁰ c for 15 minutes.

Reagent- Kovac's reagent

| Composition | Grms/Lt. |
|-----------------------|----------|
| p-diaminobenzaldehyde | 5.0 |
| Isoamyl alcohol | 75.0 |
| Concentrate Hcl | 25.0 ml |

Aldehyde was added to flask containing alcohol and it was dissolved by gentle warming to 55⁰ c in a water bath. It was stored in a dark glass bottle in a refrigerator.

Procedure

The test organism was inoculated in peptone water incubated at 37⁰ c for 48 hrs. About 0.5 ml Kovac's reagent was added and shaken gently. Formation of pink colored ring over surface layer indicated positive result.

8) Methyl Red Test:

The methyl red test is done to test the ability of organism to produce and maintain stable acid and products from the glucose fermentation and to overcome the buffering capacity of the system.

Medium- MRVP Medium (Glucose phosphate broth)

| Composition | Grms/Lt. |
|---------------------------------|----------|
| buffered peptone | 7.0 |
| dextrose | 5.0 |
| dipotassium phosphate | 5.0 |
| final pH (at 25 ⁰ C) | 6.9±0.2 |

17 grams was dissolved in 1000ml distilled water. It was distributed in test tubes in 10 ml about and sterilized by autoclaving at 15 lbs pressure for 15 minutes.

Reagent- Methyl red

| Composition | Grms/Lt. |
|-----------------|----------|
| methyl red | 0.04 gm |
| ethyl alcohol | 40.0ml |
| distilled water | 60.0ml |

Methyl red was dissolved in ethyl alcohol and water was added.

The glucose phosphate broth was inoculated with culture to be tested and incubated at 37⁰ C for 48 hrs. Methyl red indicator was to the culture and development red color indicates negative test.

9) Oxidase Test

Oxidase test is done to determine the presence of the oxidase enzyme. Oxidase reaction is due to the presence of a cytochrome oxidase system.

Oxidase reagent- 1% tetramethyl- p - phenylene-diamine dihydrochloride.

Preparation of oxidase test paper

Whatman No. 1 filter paper was cut into strips of 6-8 cm in diameter. It was soaked in the reagent till saturation. The paper strips were drained and freeze dried and stored in a dark tightly sealed bottle.

Procedure:

The oxidase test paper was moistened with distilled water. A colony to be tested was picked using glass rod and rubbed to the paper. Development of violet within the 10 seconds is an indicative of positive test.

- 10) Oxidation - Fermentation metabolism of carbohydrate and test performed as shown below:

Medium : Hugh and Leifson's oxidation fermentation medium (O/F-medium)

| Composition | Grms/Lt. |
|---------------------------------|----------|
| tryptone | 2.0 |
| Sodium chloride | 5.0 |
| diptassium phosphate | 0.3 |
| bromothymol blue | 0.08 |
| agar | 2.0 |
| final pH (at 25 ⁰ C) | 6.8±0.2 |

0.94 gm. was suspended in 100ml D/W. It was boiled to dissolved completely. It was sterilized at 15 lbs pressure (121⁰ c) for 15 minutes. To the medium sterile carbohydrate (dextrose, lactose, sucrose) solution was added to final concentration of 1%. It was mixed thoroughly and aseptically dispensed in sterile tubes.

Culture was stabbed in a pair of medium to approximately 1/4 inch from the bottom. About 1ml of sterile paraffin was added to one of the tube. Tubes were incubated were noted. Changes in color of the medium to yellow in unsealed tube indicates as changes in color of medium to yellow in both sealed and unsealed tube indicate oxidative and fermentative.

- 11) Sulphide- Indole- Motility Medium (SIM)

Sulphide-Indole-Motility is a semi solid medium used for the determination of sulfide production, Indole formation and motility of enteric bacteria.

| Composition | Grms/Lt. |
|---------------|----------|
| beef extracts | 3.0 |

| | |
|---------------------------------|---------|
| peptone | 30.0 |
| Peptonized iron | 0.2 |
| Sodium thiosulphate | 0.025 |
| agar | 3.0 |
| final pH (at 25 ⁰ C) | 6.8±0.2 |

36 grams was suspended in 100ml D/W. It was heated to boil to dissolve the medium completely. It was dispense in tubes and sterilized by autoclaving for 15 minutes at 15 lbs pressure (121⁰C). The medium was allowed to solidify in a vertical position.

Procedure:

The test organism was stabbed into the medium and incubated at 37⁰ C for 24 hrs. Motile organism diffuse growth or turbidity away from the line of inoculation and non-motile only along the line of inoculation.

H₂s positive test is indicated by blackening along the line of inoculation. 0.2 ml of Kovac's reagent was added to the tube and allowed to stand for 10 minutes. A dark red color in the reagent indicates a positive indole test.

12) Urease Test:

Urease test demonstrate the ability of an organism to spilt urea forming two molecules of ammonia by the action of the enzyme urease.

Medium- Urea agar base

| Composition | Grms/Lt. |
|---------------------------------|----------|
| peptone | 1.0 |
| dextrose | 1.0 |
| sodium chloride | 5.0 |
| disodium phosphate | 1.2 |
| monopotassium phosphate | 0.8 |
| phenol red | 15.0 |
| final pH (at 25 ⁰ C) | 6.8±0.2 |

Twenty-four grams urea agar base was suspended in 1950 ml distilled water. It was boiled to dissolve completely and sterilized by autoclaving at 101bs pressure (115⁰ C) for 20 minutes. It was cooled doen to 55⁰ c and aseptically introduced 50 ml of sterile 40% of urea solution adn mixed well. It was distributed in sterile test tube and allowed to solidify in slanted position.

Fresh culture of test organism was streaked heavily on the slant and incubated at 37⁰C for overnight, change in color of medium to pink indicate positive test and no change in color indicate negative test.

13) Voges -Proskauer Test

Vaoges- Proskauer test determines the ability of organism to produced a neutral end product, accetylmethyl carbinol from glucose fermentation.

Medium- MRVP medium

Reagent -Barritt' reagent

solution- A

| | |
|---------------------|--------|
| α - naphthol | 5g |
| ethyl alcohol (95%) | 100 ml |

Solution- B

| | |
|---------------------|----------|
| Potassium hydroxide | 40.0 g |
| distilled water | 100.0 ml |

Procedure:

Sterile broth was inoculated with fresh culture medium and incubated at 37⁰ C for 48 hrs. Development of pink- red color within 30 minutes after adding of α - naphthol and 40% potassium hydroxide in 1:3 proportion was recorded as positive test.

III) SENSITIVITY TESTING MEDIA

1) Muller- Hinton Agar

Muller-Hinton agar is used for susceptibility of micro-organism to antimicrobial agents using disc diffusion technique.

| Composition | Grms/Lt. |
|---------------------------------|----------|
| beef extracts | 300.0 |
| casein acid hydrolysate | 17.5 |
| Starch | 1.5 |
| Agar | 17.0 |
| final pH (at 25 ⁰ C) | 7.4±0.2 |

38 grams of media was suspended in 1000ml of D/W and boiled to dissolve the medium completely. It was sterilized by autoclaving at 15 lbs pressure (121⁰C) for 15 minutes, then cooled to 50⁰C and poured on sterile plates.

2) GRAM'S STAINING:

Heat fixed smear of bacterial culture was flooded with crystal violet for one minute and excess stain was washed out. The slide was treated with gram's iodide for one minute and washed. It was flooded with decolorizer acetone was

treated with safranin for 1 minutes and washed with water. It was dried and observed under microscope.

3) PREPARATION OF STAINS

a) Crystal violet

Solution - A

Crystal violet 2.0g

95% ethyl alcohol 200.0ml

Solution -B

Ammonium oxalate 0.8g

distilled water 80.0ml

Crystal violet was dissolved in ethyl alcohol and ammonium oxalate in D/W. then solution A and B were mixed.

b) Gram's Iodine

Iodine .0 g

Potassium iodide 2.0g

D/W 300.0 ml

Iodine and potassium iodide were dissolved in D/W

c) Ethyl Alcohol (95%)

absolute alcohol 5.0ml

distilled water 5.0ml

d) Safranin

Safranin (2.5% solution in

95% of ethyl alcohol) 10.0 ml

Distilled water 100.ml

APPENDIX-III

ATAGO CATALOG

TABLE 1

International Temperature Correction Table (1994)

For refractometer above and below 20°C.

| Tem °C | 0 | 5 | 10 | 15 | 20 | 25 | 30 | 35 | 40 | 45 | 50 | 55 | 60 | 65 | 70 | 75 | 80 | 85 |
|--------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|------|
| 10.0 | 0.53 | 0.56 | 0.59 | 0.62 | 0.65 | 0.67 | 0.69 | 0.71 | 0.72 | 0.73 | 0.74 | 0.75 | 0.75 | 0.75 | 0.75 | 0.74 | 0.73 | |
| 11.0 | 0.49 | 0.52 | 0.54 | 0.57 | 0.59 | 0.61 | 0.63 | 0.64 | 0.65 | 0.66 | 0.67 | 0.68 | 0.68 | 0.68 | 0.68 | 0.67 | 0.67 | 0.66 |
| 12.0 | 0.44 | 0.47 | 0.49 | 0.51 | 0.53 | 0.55 | 0.56 | 0.57 | 0.58 | 0.59 | 0.60 | 0.60 | 0.61 | 0.61 | 0.60 | 0.60 | 0.60 | 0.59 |
| 13.0 | 0.40 | 0.41 | 0.43 | 0.45 | 0.47 | 0.48 | 0.50 | 0.51 | 0.52 | 0.52 | 0.53 | 0.53 | 0.53 | 0.53 | 0.53 | 0.53 | 0.52 | 0.52 |
| 14.0 | 0.34 | 0.36 | 0.38 | 0.39 | 0.40 | 0.42 | 0.43 | 0.44 | 0.44 | 0.45 | 0.45 | 0.46 | 0.46 | 0.46 | 0.46 | 0.45 | 0.45 | 0.44 |
| 15.0 | 0.29 | 0.31 | 0.32 | 0.33 | 0.34 | 0.35 | 0.36 | 0.37 | 0.37 | 0.38 | 0.38 | 0.38 | 0.38 | 0.38 | 0.38 | 0.38 | 0.37 | 0.38 |
| 16.0 | 0.24 | 0.25 | 0.26 | 0.27 | 0.28 | 0.28 | 0.29 | 0.30 | 0.30 | 0.30 | 0.31 | 0.31 | 0.31 | 0.31 | 0.31 | 0.30 | 0.30 | 0.30 |
| 17.0 | 0.18 | 0.19 | 0.20 | 0.20 | 0.21 | 0.21 | 0.22 | 0.22 | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 | 0.23 | 0.22 |
| 18.0 | 0.12 | 0.13 | 0.13 | 0.14 | 0.14 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 | 0.15 |
| 19.0 | 0.06 | .06 | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.07 |
| 21.0 | 0.06 | 0.07 | 0.07 | 0.07 | 0.07 | 0.07 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.07 |
| 22.0 | 0.13 | 0.14 | 0.14 | 0.14 | 0.15 | 0.15 | 0.15 | 0.15 | 0.16 | 0.16 | 0.16 | 0.16 | 0.16 | 0.16 | 0.15 | 0.15 | 0.15 | 0.15 |
| 23.0 | 0.20 | 0.21 | 0.21 | 0.22 | 0.22 | 0.23 | 0.23 | 0.23 | 0.23 | 0.24 | 0.24 | 0.24 | 0.24 | 0.23 | 0.23 | 0.23 | 0.23 | 0.22 |
| 24.0 | 0.27 | 0.28 | 0.29 | 0.29 | 0.30 | 0.30 | 0.31 | 0.31 | 0.31 | 0.32 | 0.32 | 0.32 | 0.32 | 0.31 | 0.31 | 0.31 | 0.30 | 0.30 |
| 25.0 | 0.34 | 0.35 | 0.36 | 0.37 | 0.38 | 0.38 | 0.39 | 0.39 | 0.40 | 0.40 | 0.40 | 0.40 | 0.40 | 0.39 | 0.39 | 0.39 | 0.38 | 0.37 |
| 26.0 | 0.42 | 0.43 | 0.44 | 0.45 | 0.46 | 0.46 | 0.47 | 0.47 | 0.48 | 0.48 | 0.48 | 0.48 | 0.48 | 0.47 | 0.47 | 0.46 | 0.46 | 0.46 |
| 27.0 | 0.50 | 0.51 | 0.52 | 0.53 | 0.54 | 0.55 | 0.55 | 0.56 | 0.56 | 0.56 | 0.56 | 0.56 | 0.56 | 0.55 | 0.55 | 0.54 | 0.53 | 0.52 |
| 28.0 | 0.58 | 0.59 | 0.60 | 0.61 | 0.62 | 0.63 | 0.64 | 0.64 | 0.64 | 0.65 | 0.65 | 0.64 | 0.64 | 0.64 | 0.63 | 0.62 | 0.61 | 0.60 |
| 29.0 | 0.66 | 0.67 | 0.68 | 0.69 | 0.70 | 0.71 | 0.72 | 0.73 | 0.73 | 0.73 | 0.73 | 0.73 | 0.72 | 0.72 | 0.71 | 0.70 | 0.69 | 0.68 |
| 30.0 | 0.74 | 0.75 | 0.77 | 0.78 | 0.79 | 0.80 | 0.81 | 0.81 | 0.81 | 0.82 | 0.81 | 0.81 | 0.81 | 0.80 | 0.79 | 0.78 | 0.77 | 0.75 |

Temperature Correction

Calculation for Brix Reading

Mean observed refractometer reading = 15.2 (x)

Lab. Temperature of Sample = 16°C (y)

Observed refractometer reading tally with ATAGO catalogue i.e. (x)

lies between 15 to 20%

According to ATAGO catalogue, at (y). correction factor i.e. 0.27 at 15% and 0.28 at 20%.

Difference between 15% and 20% = (0.28-0.27)

= 0.01

Difference between the observed and standard percentage i.e. 15.2 -15 = 0.2%

Difference at 5% is 0.01

Difference at 1% is $0.01 \div 5 \times 0.2 = 0.0004$

\therefore The correction factor = $0.27 + 0.004 = 0.02704$

Hence , total sugar concentration = $(X - 0.2704) = 14.92$

Note : (If temperature of sample lies between 10- 19°C, minus the correction factor with observed refractrometer reading, while temperature above the 20°C, plus the correction factor.)