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(NEFOSTA)

NEPAL FOOD SCIENTISTS AND TECHNOLOGISTS ASSOCIATION

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The Association is a professional and educational organization of Food Scientists and Technologists with its central office in Kathmandu.

There are two chapters- Purbanchal (Eastern Development Region) and Narayani (Mid Development Region) of the Association, which are located at Dharan and Hetauda respectively.

Objectives

- Facilitate for the development and propagation of knowledge of Food Science and Technology.
- To provide a forum for discussion and exchange of the outcomes of research work in the field of Food Science and Technology.
- Create supportive environment in order to encourage innovative works in Food Science and Technology.

Major Activities

- Publication of Journal of Food Science and Technology Nepal
- Arranging lectures and seminars on different aspects of Food Science and Technology for the benefit of members and the Public

Membership

- Membership is open to graduates in Food Science and Technology as well as to those engaged in these professional activities. Students studying in the related graduate course can be illegible to the student membership.
- Types of membership include Life membership, General membership, Academic membership, Sub-membership, Student membership and Affiliated (Large Scale Industry, Medium Scale Industry, Small Scale Industry and Cottage Scale Industry) membership.

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Editorial

How to conceptualize a process of development? Is it a physical dimension of society? What it comprises in totality? In real sense, to conceptualize a phenomenon of social process of development has been a complex matter to all of us who have at least a feeling of responsibility in the society. We have come across a hardship of limited resources and it has been a deep-seated habit for most of us that we seek development outwardly in physical parameters. Our sense is catchy to a dimension which has acquired a physical shape. However, the earth where we are created by virtue of the evolutionary process of development and the diversities and extremities manifested in many ways indicate that the human being is more competitive and sense of supremacy has led him losing the sense of mutuality and rationalism. Now the time has come to us urging that sense of coexistence and mutuality must prevail in development and it is not a territorial concern, it has to be a global and universal concern. We have to redefine our role. Let us divert our effort to narrow down the gap between our children who are living at two extreme situation of overfed and underfed. If we claim that the stage of development has attained a significant height of contemporary time then the claim should incorporate the right of food of a hunger child and ill fed mother in the surrounding of humanity. In this regard, the role of a food scientists, food technologist and nutritionist has to be a role of global concern. The effort has to be diverted towards utilizing every available resource for the development of sufficient food, better food, with a scheme of rational distribution to all maintaining the beauty and diversity of nature. Therefore, the profession of Food Science and Technology has been more responsible and challenging than ever before. The realization should come to us and we have to share the idea and knowledge through various forum. The process of learning is a never ending one and Nepal Food Scientists and Technologists Association (NEFOSTA) request to all fellow professionals that let us endeavor our effort to the process of dissemination whatever we have deserved through our profession. As a participation to the journey, NEFOSTA has began to forward its first step by the name of Journal of Food Science and Technology Nepal (JFSTN) and it will try to accommodate the deeds and innovations accomplished by the fellow professionals in the line of Food Science and Technology. The Publication is dedicated to all who have inspired the previous and present generation of the world to make Food Science and Technology a discipline of learning and innovation for the betterment of our earth at large.

Ganesh Dawadi
Editor-in- Chief

NEFOSTA PUBLICATIONS

- Journal of Food Science and Technology Nepal (JFSTN) - An annual publication of NEFOSTA Central Executive Committee.

- Food Nepal - An annual publication of NEFOSTA- Narayani Chapter

- NEFOSTA News-Letter - Quarterly publication covering the NEFOSTA activities

- NEFOSTA-PC News-Letter- Quarterly Publication covering NEFOSTA Purbanchal Chapter activities.

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REVIEW ARTICLE
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Folates: A Review on Stability, Bioavailability, Fortification and Analysis

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Folate plays a key role in many reactions of the metabolism of amino acids and nucleotides. Deficiency of folates in the diet has been linked to anaemia, congenital neural tube defects and cleft palate, stroke, coronary diseases, and some forms of cancer. There is a good evidence of folate deficiency in a significant number of populations, prompting widespread fortification of foods with folic acid in developed countries. Folate exists in multiple forms and is highly sensitive to high temperature, aerial oxidation and light. Folate metabolism in humans with special reference to bioavailability of folic acid and its derivatives, from synthetic to food sources, is not well understood. This paper reviews current literature on the stability of folate to processing conditions with reference to rice and the bioavailability of folates. Considering low level of folates in cereal foods, particularly rice, various methods of folic acid fortification in these foods is discussed. The current information available on extraction, purification and detection of folates in foods, and how it can be used to improve the folate analysis is also summarized.

Keywords: Folates, Property, Stability, Bioavailability, Fortification, Analysis

Introduction

Folate is the term most commonly used to refer to a family of vitamers with related biological activity. The term folic acid and folate or folacin is often used interchangeably. While folate refers to all forms of the vitamin including the naturally occurring forms of the vitamins (polyglutamates), folic acid refers to the most oxidized, stable, and easily absorbable synthetic form (monoglutamate) (Gregory, 1989). For a long time folate deficiency in the diet has been linked with megaloblastic and macrocytic anaemia, along with Vitamin B12.Various studies and randomised trials over the last three decades have shown that adequate intake of folate reduces the risk of abnormalities in early embryonic brain development and specifically the risk of malformations of the embryonic brain/spinal cord, collectively referred to as neural tube defects (NTDs) (MRC, 1991; Czeizel & Dudas, 1992). Various investigations have also linked low level of folates with increases in plasma homocysteine level, which is an independent risk factor for occlusive vascular disease and stroke (Selhub *et al.*, 1993); development of certain cancers such as colorectal and uterine cervical (Glynn & Albanes, 1994); and more recently breast cancer (Zhang *et al.*, 1999); Alzheimer's (Wang *et al.*, 2001); and cleft palate (Shaw, Zhu & Lammer 2003). In an effort to increase the folate status of child-bearing women and hence reduce the occurrence of neural tube defects, several countries have introduced mandatory or voluntary folate fortification programs.

It is unknown what effect these current levels of folate exposure may be having on the population. In order to understand whether the current fortification levels require amendment, the main questions to be answered are; what the effect of processing is on folate content in food; apparent consumption of specific foodstuffs; how much of the folate in these specific foods is being absorbed and what effect (if any) are these exposure levels having on health and disease prevention?

Analysis of folate is not easy due to its multiple forms, lower stability, and occurrence in lower concentrations in biological systems, and complex extraction and detection techniques. Accurate methods of folate analysis in foods are needed for food composition, nutritional labelling, nutritional intervention studies and planning diets.

Another important area of folate research is its bioavailability. Understanding folate bioavailability and its assessment is both complex and difficult to achieve due to the variability of folate metabolism and function between individuals complicating the measurement of folate bioavailability in humans for any one food (Gregory, 1995). This review assesses the current literature on stability, bioavailability, fortification and analytical techniques for folates.

Properties and sources of folates

The fundamental unit of folic acid is made up of a bicyclic pterin linked by a methylene bridge (C^9—N^{10}) to para-aminobenzoic acid (pABA), which is joined by peptide linkage to a single molecule of L-glutamic acid (Figure 1). The pteridine moiety of folates can exist in three oxidation states: fully oxidized (folic acid); partially reduced 7, 8-dihydro (H_2 folate); or fully reduced 7, 8, 9, 10-tetrahydrofolate (H_4folate). Tetrahydrofolates are coenzymic forms of the vitamin. In

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nature, these folic acid derivatives exist as γ-linked polyglutamate conjugates, with chain lengths in the range of five to seven residues. Over 100 derivatives of folic acid may exist naturally due to this chemical diversity (Gregory, 1989). Due to their structure, folates in foods have been traditionally reported as "free" and "total folate" values. The "free folate" values indicate the *L. casei* activity measured without enzymatic deconjugation where as "total folate (or folate only)" values mean the *L. casei* activity with enzymatic deconjugation (Tamura & Stokstad, 1973). The "free folate" is now commonly referred to as "undeconjugated folate".

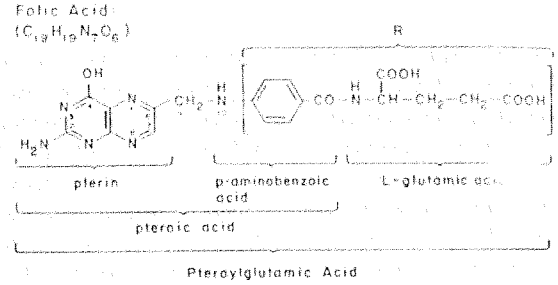

Figure 1: Structure of folic acid
(Source: Hawkes and Villota, 1989)

Folic acid (PGA) is a yellow crystal with a molecular weight of 441.4. It is the form that is used for the folate fortification in foods. It has limited solubility in water and is insoluble in organic solvents such as alcohol, ether, and benzene (Lund, 1994). Folic acid is more stable in alkaline than acidic conditions, thus standards for folic acid derivatives are prepared in basic solution (Keagy, 1985). The major sources of folates are green leafy vegetables, liver, beans and legumes, egg yolk, wheat germ, and yeast and its products. Our study showed soybean as one of the excellent sources of folate with folate content of about 400 μg per 100 g (Arcot, Wong & Shrestha, 2002; Ginting, Arcot, Cox & Shrestha, 2002a & b). Pulses are also very good sources of folates too (Shrestha & Arcot, 1999). In recent years, fortified cereal products are readily available in some countries thereby contributing an important source of dietary folate.

Stability of folates

There is great variability in the thermal stability of folates (Chen & Cooper, 1979; Day & Gregory, 1983; Paine-Wilson & Chen, 1979). The degree and rate of destruction is largely influenced by the pH of the medium, reducing agents in the buffer, folate derivatives, type of buffer, and the food system. Chen and Cooper (1979) reported that tetrahydrofolic acid is extremely labile to heat compared to 5-methyl-tetrahydrofolic acid with a half-life of 2.25 min and 21.4 min at 100°C, respectively. The stability of both folate derivatives drastically increased with incorporation of ascorbic acid, thus showing the detrimental effect of molecular oxygen at higher temperature. Day & Gregory (1983) found that folic acid and 5-methyl-tetrahydrofolic acid are stable even if heated at higher temperature (up to 140°C) at low oxygen concentration. O'Broin, Temperley, Brown & Scott (1975) found folic acid to be the most stable followed by 5-formyltetrahydrofolic acid among the folate derivatives. Folic acid was the most stable of all folate vitamers; stability increased with alkalinity. Light is reported to cause cleavage at C^9—N^{10} position (Krumdieck & Baugh, 1969). Folates have been reported to have low stability at low pH (below 4.5) (Rao & Naronha, 1978; Lund, 1994). Ascorbic acid, 2-mercaptoethanol and dithiothreitol act as scavengers of molecular oxygen in folic acid solution and delay the initiation of oxidation; ascorbic acid is the most effective on a molar basis (Rao & Naronha, 1978). Ascorbic acid (1%) limits 5-methyl-tetrahydrofolic acid destruction to 20% after 3 hours at 100°C. Without ascorbate the same vitamin loses 90% of its activity after 65 minutes of boiling (Chen & Cooper, 1979). We extracted folic acid from fortified breakfast cereals with and without ascorbic acid in the buffer at 121°C for 10 min. The results showed that ascorbic acid in the extraction buffer offered significantly ($p<0.05$) better protection against oxidation (Arcot, Shrestha & Gusanov, 2002).

In comparison to the number and depth of studies undertaken to describe and understand the loss of many other nutrients during food processing, there are few published data on losses of folate. Dang, Arcot & Shrestha (2000) investigated the effect of soaking, boiling and pressure-cooking on the retention of folates in whole chickpea and field peas. About 20% of folate leached into the soaking medium for both legumes. Boiling and pressure-cooking caused a loss of about 50% folates in both legumes. Soetrisno, Holmes and Miller (1982) reported a significant loss of folates in soaked and autoclaved soybean: a loss of 36%, 33% and 28% folates after 5, 10 and 15 min autoclaving respectively. De Souza & Eitenmiller (1986) found an 83% and 42% loss of folate after water and steam blanching respectively, compared with fresh sample. Following water blanching, spinach samples subjected to canning lost a further folate content of fresh spinach. It has been shown that ionization radiation affects the folate content of plant foods. Muller & Diehl (1996) reported a loss of 10% folate in spinach, cabbage and Brussels sprout when exposed to 2.5 kGy radiation, and at higher dose of 10k kGy upto 30% total folate was lost. Arcot, Wong & Shrestha (2002) reported a loss of 9 and 16% undeconjugated and total folates respectively when raw soymilk was heated to produce UHT soymilk.

Soaking and boiling significantly reduced the folate content in soybean. But *Rhizopus* fermentation of boiled soybean, as in *tempeh* preparation, reported to increase the folate content of soybean significantly (Arcot *et al.*, 2002; Ginting *et al.*, 2002a and b). They suggested that increase in total folate compounds may be due to release of total folate present in the bound form in soybeans by *Rhizopus* enzymes or by synthesis of these compounds by *Rhizopus* itself. Arcot *et al.* (2002) further reported that a significant portion of *tempeh* folate (36%) is lost during deep frying. Our study also showed marked increase in folate content of soybean seed during soy sauce preparation (Ginting *et al.*, 2002b). During brine fermentation, we noticed a change in folate distribution, usually the predominant form of folate in soybean, 10-formyl tetrahydrofolate, superseded by 5-

methyltetrahydrofolate after brine fermentation. The folate content of milk significantly increased during fermentation *i.e.*, to yoghurt, cheese, kefir etc., due to the presence of active folate-producing bacteria (some species of *Lactobacillus, Streptococcus, Penicillium*) (Rao, Reddy, Pulusani & Cornel, 1984). Chen, Song & Kirsch (1983) investigated the effects of blanching, freezing and storage on the folate content of spinach. Folate losses of 7%, 26% and 27% were observed in fresh spinach held at room temperature for 10 h, in a refrigerator for 7 days, and in a freezer for 10 weeks, respectively. Microwave blanching and quick-freezing caused only 14 to 16% loss of folate, but water blanching doubled the loss of folate to 28%.

Folate metabolism

Food folate mainly consists of reduced polyglutamates which are hydrolysed to monoglutamates in the gut prior to absorption across the intestinal mucosa. The hydrolysis is carried out by γ-glutamyl-carboxypeptidase (folate conjugase), present primarily in the lumen and in the brush border of the intestine (Reisenauer, Halsted, Jacobs, & Wolfe, 1985). Dietary folates are absorbed as folic acid, 5-methyl-tetrahydrofolic acid, and 5-formyltetrahydrofolic acid. However, the predominant form in portal plasma is the reduced form, tetrahydrofolic acid. Transport of folate is complex and poorly understood. It is assumed that dietary folates, after hydrolysis, reduction and absorption from the intestine, are transported in plasma to liver, which releases it to the peripheral plasma after it is converted primarily to 5-methyl-tetrahydrofolic acid, but also to 10-formyltetrahydrofolic acid. The plasma concentration of 10-formyltetrahydrofolic acid is tightly regulated whereas that of 5-methyl-tetrahydrofolic acid is not; thus the latter varies in response to folate meals (Basu & Dickerson, 1996). It is believed that most of the monoglutamate form of folates is converted into 5-methyl-tetrahydrofolic acid in enterocytes. About 50% of this folate is stored in the liver and distributed to peripheral tissues via enterohepatic circulation. These folates are taken up by cells and are metabolised intracellularly to polyglutamate forms (Comb, 1998).

Folates act as a coenzyme substrate in many reactions of the metabolism of amino acids and nucleotides. The majority of naturally occurring folates, when ingested, are converted to 5-methyl-tetrahydrofolate. The 5-methyl-tetrahydrofolate transfers its methyl group to homocysteine by the vitamin B_{12}-dependent enzyme methionine synthetase reaction, generating tetrahydrofolate and methionine. Without adequate supply of vitamin B_{12} to accept the methyl group homocysteine accumulates at the expense of the other metabolically active folate pools. Methionine resulting from methylation of homocysteine is an essential amino acid for the synthesis of proteins and polyamines. It also acts as a precursor for S-adenosylmethionine, which serves as a donor of methyl groups for more than 100 enzymatic reactions that have critical roles in metabolism. Methionine is also essential for the initiation of protein synthesis (Mills *et al.*, 1996).

The presence of genetic defects in normal folate-dependent metabolic processes involving DNA synthesis has been linked to cancer initiation (Fenech, Noakes, Clifton, & Topping, 2001). Several studies establishing the relationship of folate to DNA methylation, DNA repair, and oncogene expression have provided the basis for the hypotheses relating folate nutriture to cancer risk and prevention (Selhub & Rosenberg, 1996). The relationship between folate status and colorectal cancer is more convincing than its relationship with other forms of cancer (Bailey, 1995).

There are three major forms of NTDs: spina bifida, anencephaly and encephalocele. Spina bifida results from the failure of the spinal column to close during the 24th and 27th day of pregnancy, usually before a woman is aware that she is pregnant (Hoffpauer, 2004). Though most of the babies survive, their lower limbs are affected in varying degree of severity, resulting in paralysis as well as poor bladder and bowel control. In severe cases, babies may also have hydrocephalus (a condition that requires surgery to relieve excess fluid pressure on the baby's brain). Some children with spina bifida and hydrocephalus also have mental retardation. Anencephaly is another defect characterized by the incomplete development of the bones of the skull and a partially or completely absent brain. Most babies with this disease are stillborn, and live babies with this condition survive only for short periods, usually dying within the first week of birth (Mulinare 1995). In the United States, Canada, and Australia, the occurrence of NTDs is about 10 births per 10,000 (Australian Institute of Health and Welfare, 2000). Although data for NTDs and spina bifida is not available for Nepal, a significant number spina bifida cases has been reported in Nepal (Baskota, 2004).

The Recommended Dietary Intake (RDI) for folate is 200μg for normal adults, 400μg for pregnant women and 350μg for lactating mothers (NH&MRC, 1995). The upper safety limit of folate intake is set at 1 mg per day based on the scientific evidence of possible negative effects caused by high dose of folate such as the masking of vitamin B_{12} deficiency (NH&MRC, 1995).

Bioavailability of folate

In order to understand folate bioavailability and its assessment in the healthy body; it is important to understand bioavailability to be the net result of release from the food matrix, uptake via the brush border, conversion into bioactive forms (including deconjugation of polyglutamates), active transport, diffusion into cells and plasma and excretion via renal, hepatic and faecal routes. Thus, the understanding of folate bioavailability and its assessment is both complex and difficult to achieve. In addition, the variability of folate metabolism and function between individuals complicates the measurement of folate bioavailability in humans for any one food. In spite of numerous studies, our understanding of folate bioavailability is still incomplete, and it is not possible at this time to predict the bioavailability of folate for a given diet (Gregory, 1995). This is supported by the large variability in folate bioavailability values

for the same food in different individuals (Tamura & Stokstad, 1973; Babu & Srikantia, 1976). Bailey (1988) reviewed some of the factors that affect the bioavailability of folate in humans: polyglutamate absorption; intraluminal pH; conjugase inhibitors; food matrix; milk folate-binding proteins; thermal processing; folate form; drugs and alcohol; ageing; and nutritional deficiencies. Considering the complexity of folate structure, absorption and excretion phenomenon, the results of bioavailability studies of folates seem to be largely governed by the methods applied. *In vitro* studies of the effect of human intestinal conjugase on hydrolysis of folate polyglutamate and subsequent absorption in human body have been tried. But the process found to be too complex and did not yield any useful information (Scott, Rebeille & Fletcher, 2000). Animal studies are frequently used in bioavailability of various nutrients. However, very few bioavailability studies of folates have been performed on animals, such as one carried out by Clifford, Heid, Muller & Bills (1993) on rats. Due to the large differences that exist in the process of digestion and absorption of folate between animals and humans, adaptation of their model to humans is questionable. Other common methods of folate bioavailability in humans are plasma kinetics, urinary excretion and feeding experiments.

Plasma kinetics: After the ingestion of a water-soluble substance, the concentration of that substance in the plasma is elevated for a certain time period until the processes of cellular accumulation and/or excretion return of the plasma concentration to its initial baseline. Multiple blood samples during this period can be used to produce an area under the plasma concentration versus time curve (AUC). AUC profiles based on total folate measurement of the plasma have been used (Prinz-Langenohl, Bronstrup, Thorand & Hages, 1999; Fenech *et al.*, 1999) however they provide little information about the fate of the administered dose because the measured folates may involve those newly absorbed, from previous dietary intake or body pool turnover. Several studies have found that the time for maximal plasma concentration to be reached is between 1 and 2 hours postprandially (Fenech *et al.*, 1999) independent of the test dose or energy intake. Hence the change in the plasma folate concentration that occurs 1 - 2 hours after eating a folate-rich meal has also been used as a short-term indicator of available folate (Colman, Green & Metz, 1975a & b). However, this method relies on the assumption that the rate of 'folate absorption in the test and reference doses are similar.

Plasma kinetics have been extensively used to study bioavailability of folic acid (and also monoglutamates) compared to it being given as a polyglutamate. There are some studies that agree with equal utilization of the mono- and polyglutamyl forms (Tamura & Stokstad, 1973; Bailey, 1988) whereas others have shown that polyglutamate folate is utilized 70-80% as efficiently as the monoglutamate form (Keagy, Shane & Oace, 1988). Colman *et al.* (1975a) found that synthetic folic acid in cooked food may be absorbed at a lesser rate than that administered in water. The test based on a short term change in serum folate showed that the bioavailability of folic acid added to maize (as porridge) or rice was about 55%, while the bioavailability of folic acid added to bread was approximately 30%. Colman *et al.* (1975b) also showed that maize meal containing a daily dose of 500 µg folic acid produced an effect similar to that of 300 µg daily in tablet form. These findings support the efficacy of the fortification of cereal-grain products as a means of distributing available folate to target population. There are conflicting reports on bioavailability of folic acid *vs* reduced folates too.

Urinary excretion: The urinary excretion of folate is proportional to the serum folate concentration under the assumption that the urinary clearance of folate is constant and thus is often used as a measure of folate availability (Keagy *et al.* 1988). It uses extensive saturation of the tissues to the point where folate accumulates in the plasma and is excreted in the urine (Scott *et al.*, 2000). In essence, a timed urine collection represents a timed integral of the AUC and is easier and less expensive than the AUC but subject to lower precision, larger individual variation and less sensitivity as renal clearance "adds another layer of biological variability" (Heaney, 2001). This approach has also been used to compare bioavailability of differing folates monoglutamates compared to folic acid (Scott *et al.*, 2000). Tamura & Stokstad (1973) studied the availability of naturally occurring folates from a number of foods based on urinary excretion. The authors found, among 12 food items, banana, lima beans, liver and brewer's yeast contained relatively high available folates, whereas orange juice, romaine lettuce, egg yolk, cabbage, defatted soybean and wheat germ contained poorly available folates. Babu & Srikantia (1976) also found considerable variation in the bioavailability of folate from different foods: 72% for egg, 70% for liver, 70% for Bengal and Green gram, 63% for spinach, 50% for banana, and 10% for brewer's yeast.

Long-term feeding protocols: Long term protocols, typically one to two months, are generally more suitable for comparison of diets that differ in their sources of folate (foods naturally high in folate versus fortified foods) rather than single food items (fortified bread versus a folic acid supplement) due to the difficulty in maintaining subject interest and compliance and the possible interactions that mixed diets can have on the bioavailability of folate in a single food. The basic principle is that if the total folate content of different diets is similar then any difference in the folate status of the individuals is due to a difference in the bioavailability of the folate within that diet. Cuskelly, McNulty & Scott (1996) conducted a long term feeding trial that indicated the bioavailability of folic acid fortified foods (bread and breakfast cereals) as not being significantly different from that of a folic acid supplement. The study also showed that the bioavailability of added folic acid was greater than that of naturally occurring food folate. Various researchers have found that there is no effect of dietary fibre and complex carbohydrates on bioavailability of monoglutamates in humans (Ristow, Gregory & Damron, 1982; Keagy & Oace, 1984). However, the negative effect of wheat

bran on the bioavailability of polyglutamyl folate has been reported by others (Bailey *et al.*, 1988; Keagy *et al.*, 1988).

One of the difficulties with the bioavailability study of nutrients is the difficulty in discriminating between physiological nutrient levels present in the body at any time and post-absorption concentrations following a recent oral dose. Recent practice of using stable isotope labelled folic acid (or other vitamers) is beneficial in the fact that it can specifically follow the metabolic and physiological fate of the administered dose while not posing any health concerns to the subjects. This procedure now expected to produce more accurate and reliable results on the fate of food folate (added) in human body and improve our understanding of folate bioavailability. Folate can be labelled by incorporating a stable isotope of any of the constituent elements (^{2}H, ^{13}C, ^{15}N, or ^{18}O) into the molecule, the two most commonly used being ^{13}C and ^{2}H. It is essential in isotopic studies that the labelling is present in a metabolically and chemically inert part of the molecule throughout the entire processes of metabolism, excretion and analysis in order to ensure that there is no loss of the label. For this reason labelling of the folate molecules has mainly taken place in either the para-aminobenzoate moiety or the first amino acid glutamate moiety. A comprehensive review on the development of stable isotope synthesis has been published by Gregory, Bailey, Toth, & Cerda (1990).

Folate fortification

Three possible means to increase the availability of folates in women of child-bearing age have been identified: 1. encouraging dietary improvement, 2. promoting the use of supplements containing folic acid, and 3. fortifying the food supply. Staple food fortification is seen to be the most effective means of reaching the majority of women, whether they are planning a pregnancy or not, and does not involve a change in eating behaviour. It is now a common practice to fortify cereal products with folic acid in countries such as the USA, Australia, UK, Canada and Israel. Although fortification has been regarded as the best method to meet the folate requirement of the target population, FDA acknowledged that there is the risk of exposing some groups of population, causing potential adverse effects. The most documented study is a masking of clinical symptoms of pernicious anaemia in persons with vitamin B_{12} deficiency while irreversible neurologic damage progresses. The groups at potential risk from increased intakes of folate include those on anti-seizure or anti-cancer medications that interfere with folate metabolism, pregnant women, and children, for whom a safe upper limit has not been determined (Yetley & Rader, 1995). In addition, few studies have found that prolonged intake of folic acid intake is also associated with multiple births (Muir, 2001).

The level of haematological folate values in US population have risen significantly after folate fortification has been made mandatory and incidence of NTDs have reduced significantly too, up to 19%, indicating that the fortification scheme has been successful in reaching a significant sector of the US community (Margaret *et al.*, 2001). There is an ethical issue associated with folic acid fortification. Food fortification supplies more folate to those who eat most food. This group (young men) is at the least risk of folate deficiency. The populations most in need of folate (young women) are those who eat the least food in terms of of quantity. Hence the question arises: *is it ethical to give a high folate dose to one part of the population, which does not need it to benefit another part of the population which does?*

Fortification of foods

Fortification of cereals and its products: Cereals and its processed products have been extensively used for folic acid folic acid. It is now common in developed countries to fortify bread, pasta, breakfast cereals with vitamins and minerals including folic acid. Among other foods, rice has been regarded as an ideal food for folic acid fortification due to a naturally low folate level in milled rice and its widespread consumption, particularly in Asia. Brown rice is considered to be a good source of vitamins and minerals including folate, but the milling remove a large fraction of it, resulting in milled rice low in vital nutrients (Juliano & Bechtel, 1985). Our analysis of long grain milled rice showed folate content of 41μg per 100 g (Shrestha, 2003). Further, the practice of washing and cooking of rice in excess water (and draining) deplete the folate level in milled rice. We found that rinsing of rice with water for 60s caused a loss of 68% folate whereas boiling of rice in excess water for 30 min caused a loss of 96% folate (Shrestha, Arcot & Paterson, 2003). Fortification of rice with water soluble vitamins is a better and more convenient method of replenishing or increasing the nutrient content of rice than under milling and parboiling. Rice fortification has been in place since the late 1940s. Most of the fortification of rice is generally done by grain-type enrichment where rice kernels are first treated with vitamins and minerals concentrate to develop a 'vitamin premix' which is then blended with white rice in the proportion of 1:100 or 1:200 to yield recommended level of nutrients (Hoffpauer, 2004). Most of these methods, such as Hoffman La Roche-Mickus method (Furter & Lauter, 1949); Rice Growers Co-operative Limited, Australia method (Bramall, 1986); Shingen method (Hunnel *et al.*, 1985); and Cross-linked grain (Joseph, Luizzo & Rao, 1990) uses acids and are not suitable for folic acid fortification (as reviewed by Shrestha, 2003). Since folic acid is less stable at low pH, these methods are unsuitable for folic acid fortification of rice.

We tried more than 50 formulations of edible coating solutions (individual or composite) based on cellulose derivatives, starch derivatives, various gums, rice protein concentrate and alginates on to the rice kernels previously sprayed with folic acid solution. A rice premix of about 40 mg (40,000 μg) folic acid was prepared, to be diluted at 1:200 ratio with milled rice. A loss of 5 to 72% folic acid during washing of fortified rice premix was noted: highest retention was in ethylcellulose coated rice followed by locust bean gum, agar and pectin and their composite mixtures. None of the coating material could prevent the loss of folic

acid while cooking fortified rice premix in excess water. The yellow colour of folic acid could not be masked by any of the coating solution and colour masking agents such as calcium phosphate; rice starch powder etc. had to be used. However, sensory evaluation of cooked fortified rice showed that there were no differences between cooked fortified rice and the untreated cooked rice (Shrestha et al., *2003).*

Knowledge of the stability of folate in food is important for the fortification program. Our research has shown that total folate content of unfortified and fortified bread samples increased from the flour to the fresh dough stage, and continued to increase significantly ($p<0.05$) as proofing of the dough occurred whereas baking caused a loss of 66 % total folate in unfortified bread and 59% and 41% in fortified breads (Arcot, Wootton, Alury, Chan & Shrestha, 2001; Chow, Arcot, Paterson & Shrestha 2001). However, Cort, Borenstein, Harley, Osadca & Scheiner (1976) reported high stability of folic acid during bread baking. Ranhotra, Gelroth, Novak & Matthews (1985) reported a loss of slightly more than 20% folate in cooked pasta products such as, spaghetti, noodles, and macaroni.

Folate analysis

Because of the increasing significance of folates in health or disease, there is a need for the development and optimisation of the methods that give more reliable folate content in foods. This section reviews the existing methods of folate analysis in foods, with special attention to extraction techniques. Methods of analysis for folic acid and its analogues are grouped into biological, microbiological, bio-specific procedures (radioassay), chromatographic, and chemical methods. Although biological method using chick and rat assays were used before, they are no more used for folate except in studying nutritional aspects of the vitamin (Keagy, 1985). Chemical methods are rarely used for routine folate analysis in food. A summary of strengths and shortcomings of microbiological, chromatographic (HPLC), and bio-specific procedures is given in Table 1. The method to be used for a particular biological material depends on the nature and purpose of the assay. Moreover, the sample preparation and extraction methods largely influence the amount of folate present in the extract, which is then assayed by a method of choice.

Table 1: Some attributes of principal methods used for folate analysis

Principle	Key steps	Advantages	Limitations
Microbiological assay (MA)			
The amount of growth of the folate dependent microorg-ananism is proporti-onal to the amount of folate in the medium. Growth measured by change in solution turbidity.	Extraction of folate from matrix Deconjugation Growth of micro-organism Measurement of turbidity	Low equipment set up costs Versatile, similar response to most folate isomers Can measure mono- to polyglutamates ($n\geq3$) Very sensitive, measure up to sub-nanogram levels 'Gold standard' for folate analysis	Test organism may be stimulated or inhibited by non-folate substances Tedious and time consuming Requires microbiological expertise Microorganism finicky and needs proper transfer and maintenance
High performance liquid chromatography (HPLC)			
Chromatographic separation of folate isomers followed by quantification, based on detector response against standards	Extraction and deconjugation of folate Purification of extract Chromatographic separation of folate isomers Detection and quanti-fication against folate standards	High specificity towards folate isomers Proven, reliable technology Unambiguous identification of isomers Minimal interference from food enzymes	High equipment set up cost Requires standards for all isomers Low sensitivity, (detect up to µg levels) Not suitable for di- and polyglutamates Yields lower total folate value than MA
Enzyme protein binding assay (EPBA)			
Competitive reaction between folate isomer and folate binding protein for a limited number of specific binding sites A bio-specific procedure	Extraction of and deconjugation of folate Reaction of folate with folate-binding protein in microtitration plate React with substrate Measurement of colour developed	Rapid (1-3 h) No equipment set up cost, relatively inexpensive No expertise needed, easy to perform High specificity towards folate isomers	Considerable variation between different kits Self-life of kits very short Not suitable for di- and polyglutamates Response between individual monoglutamates may vary

Sample preparation and purification: The sample preparation step has received the least attention, although it can have a significant effect on availability of folate for deconjugation and subsequent detection. Most often, sample preparation involves grinding of sample and homogenisation of ground food in a suitable buffer system followed by heating and centrifugation. Heating during the extraction procedure causes thermal denaturation of folate-binding proteins and enzymes that may catalyse the folate degradation or interconversion and at the same time also precipitates structural proteins (Gregory, 1989; Keagy, 1985). A range of temperatures, from 70°C to 121°C, have been used to heat the homogenate (Phillips & Wright, 1983; Pffeifer, Rogers & Greogory, 1997; Rader, Weaver & Angyal, 1998). Vahteristo, Ollilainen, Pekka & Varo (1996) reported that heating of the sample by a microwave produces results similar to the boiling water bath extraction. It is more or less established that heating destroys the folic acid derivatives but the degree and rate of destruction is largely influenced by the pH of the medium, reducing agents in the buffer, folic acid derivatives, type of buffer, and the food system itself. Our study in fortified breakfast cereals showed that although the extraction method that uses autoclave (121°C) may release more folate than a boiling water bath, the results were in slight favour of the latter (Arcot *et al.*, 2001). This could be due to the fact that unlike most of the foods where folates are bound to the macromolecules forming a complex food matrix, added folic acid in fortified foods might have only been physically bound which could be released by simple boiling. Therefore, it is difficult to suggest a particular heating condition for extraction as food matrix largely determines the release, and also, susceptibility of folate in food to heat (Shrestha, Arcot & Paterson, 2000; Tamura, Mizuno, Johnson & Jacob, 1997).

The use of 1% ascorbic acid at the pH of about 6.0 found to be satisfactory (Phillips & Wright, 1983; DeSouza & Eitenmiller, 1990; Rader *et al.*, 1997; Tamura *et al.*, 1997) for folate extraction. Keagy (1985) and Gregory (1989) reported the pH of the buffer may be 4.5-7.85, depending upon the pH optima of the enzyme used for subsequent deconjugation. There is very little research on the effect of buffers and pH of the extraction efficiency on the stability of food folates during extraction.

The extraction technique also affects the analysis of food folates. Gregory (1989) reported that a double extraction procedure (i.e., residue from the first extraction re-suspended and centrifuged) yields more folate than a routine single extraction procedure (Gregory, 1989). Our study (Shrestha *et al.*, 2000) showed that heating and centrifugation of food homogenate before tri-enzyme treatment (deconjugation) causes lowering of total folate in test foods such as spinach, fortified bread and breakfast cereals. It is believed that sensitivity of folates to high temperature and the removal of undigested food residues after centrifugation, which could have entrapped some folates, might be responsible for the decreased values.

Enzymatic deconjugation of folate: Most natural folates occur in the polyglutamate forms. *Lactobacillus rhamnosus* responds equally through mono- to tri-glutamate but much more slowly to longer-chain derivatives (Tamura et al., 1972). Similarly, bio-specific techniques and HPLC do not respond well to longer-chain derivatives of folates. Therefore, conversion of polyglutamates to mono- or diglutamate requires γ-glutamylcarboxypeptidase (conjugase or folate hydrolase). Once hydrolysed, these folates can support the growth of *L. rhamnosus* or be quantified by other methods. Chicken pancreas and hog kidney are the most commonly used sources of the conjugase whereas human and rat plasma, rat pancreas, and rat liver have been used to a lesser extent (Keagy, 1985). The activity of these conjugases differs in their pH optima, mode of action, and type of folate product tested (Kirsch & Chen, 1984; Gregory, 1989). Human plasma, hog kidney and rat plasma are reported to give monoglutamates as an end product which make them suitable for use in HPLC and bio-specific assays (Goli & Vanderslice, 1992; Keagy, 1985).

Desiccated chicken pancreas conjugase, commercially available, contain very high endogenous folate and significant level of amylases (Pederson, 1988; Shrestha *et al.*, 2000). The chicken pancreas conjugase is an exopeptidase with a pH optimum of 7.8 and produces a diglutamate end product (Goli & Vanderslice, 1992). The concentration of chicken pancreas, pH and incubation time for deconjugation were found to vary with investigators, for example, deconjugation time for chicken pancreas conjugase was also found to differ in various studies from 2 h to overnight (Kirsch & Chen, 1984; Tamura *et al.*, 1997; Martin, Landen, Soliman & Eitenmiller, 1990; Rader *et al.*, 1998; Shrestha *et al.*, 2000).

Tri-enzyme extraction: Eitenmiller and colleagues (De Souza & Eitenmiller, 1990; Martin *et al.*, 1990) reported a method of folate extraction where, in addition to the traditional treatment with folate conjugase, protease (EC 3.4.24.31) and α-amylase (EC 3.2.1.1) were also used. The extraction method was named as the "tri-enzyme treatment". It was intended to accomplish a more complete extraction of folates that may be trapped in or bound to the matrices of protein and polysaccharides, by using protease and α-amylase in addition to the heat treatment and the conjugase. De Souza & Eitenmiller (1990) observed a remarkable increase in folate values when results from tri-enzyme extracts were compared with a single enzyme in rye bread (500% increases) and in beef flank (340% increases). In a similar study, Martin *et al.* (1990) found a mean increase of 19% folate values in 12 food products of diverse matrix after tri-enzyme treatment. These two studies showed that traditional conjugase treatment does not completely free folate from a complex food matrix before folate analysis. Recent investigations have further confirmed the effectiveness of tri-enzyme technique over traditional single enzyme extraction in various food items (Pfeiffer *et al.*, 1997; Tamura *et al.*, 1997; Rader *et al.*, 1998; Shrestha *et al.*, 2000). Although the order of enzyme addition was found to differ with investigators, the more common order appeared to be protease, α-amylase and finally conjugase (Tamura *et al.*, 1997; Rader *et al.*, 1998). The food extract treated with the tri-enzymes was clearer and less viscous as compared to conjugase-only treated extract that helps in purification and yields better peak shape in HPLC analysis (Pffeifer *et al.*, 1997).

However, it appears that the number and type of enzymes used in the extraction are largely determined by the nature and composition of foods. For example, the use of amylase in meat and meat products and protease in starchy foods is not justifiable. A study by Iwatani, Arcot & Shrestha (2003) and Shrestha *et al.* (2000) showed no significant difference in folate content of vegetable extracts treated with tri-enzyme and a single enzyme (chicken pancrease). It is now realized that conditions of the enzyme treatment might be different for each type of food and therefore, the identification of the optimum pH and a suitable incubation time for each food must be done prior to folate analysis (Shrestha *et al.*, 2000).

Folate assay

Microbiological assay: The realization of the fact that certain microorganisms require specific nutritional factors that they are unable to synthesize themselves led to the application of these microorganisms to the quantitative determination of vitamins in the early 1940s. Despite the advent of several alternative methods for determining folates in foods and biological samples, the microbiological assay using *Lactobacillus casei rhamnosus* (ATCC 7469) in casein based media remains the standard method for most applications. *L. rhamnosus* is the most commonly used assay organism for folate analysis because it responds to the widest variety of folate derivatives, including 5-methyltetrahydrofolate, the predominant folate form in plasma, red blood cells and liver, and the formyl derivatives (Krumdieck, Tamura & Ito, 1983).

Traditional microbiological assay of folate that uses serial transfer of *L. rhamnosus* is difficult for routine laboratory analysis owing to frequent variations of the inocula, the requirements for microbiological expertise, and facilities for the maintenance of bacterial culture given that these conditions are not easily met in the usual clinical-nutrition laboratory and takes longer time (3-4 days). However, the use of glycerol cryoprotected frozen inoculum of *L. rhamnosus* reduces the cost and time of the assay, gives better reproducibility and makes it less tedious than using the conventional serial culture (Wilson & Horne, 1982; Shrestha *et al.*, 2000). Many laboratories also use chloramphenicol-resistant strains of *L. rhamnosus* and chloramphenicol-supplemented media that eliminate the need for heat-sterilization, centrifugation, and sample dilution (O'Broin, Scott & Temperly, 1973). It is reported to give higher folate content and also do not need ascorbic acid in the buffer to prevent folate oxidation (O'Broin *et al,* 1973). Microbiological assay of folate has also been automated using 96-well microtitre plates that takes shorter time, use less reagents, and less laborious (Newman & Tsai, 1986).

High performance liquid chromatography (HPLC): The limitation of *L. rhamnosus* and other assay organisms in differentiating folate derivatives in the folate extract has prompted the use of chromatographic techniques. These techniques involve two distinct steps: separation and purification of deconjugated extract, and detection and quantification of eluted monoglutamates (Gregory, 1989).

Ion-exchange chromatography is a popular technique for separation and purification of individual folates. Purification of extracts has been performed by using small columns of DEAE-Sephadex A-25 (Gregory, Sartain & Day, 1984); weak anion-exchange column (Goli & Vanderslice, 1992); a strong anion exchanger (Vahteristo *et al.*, 1996); and cation-exchanger (Duch, Bowers & Nochol, 1983). The biological specificity of folate binding proteins (FBP) has also been applied to purification of folate compounds (Selhub, Ahmad & Rosenberg, 1980). Several HPLC analyses of various extracts used affinity chromatography columns prepared with immobilized FBP from milk (Pfeiffer *et al.*, 1997).

The low concentrations of folate present in most foods or other tissues limit the applicability of any separation technique and emphasize the need for sensitive detection techniques (Vahteristo *et al.*, 1996). Some commonly used detection methods for eluted folate derivatives are ultraviolet absorbance (280 nm), fluorescence, and electrochemical techniques, and microbiological assay of collected fractions (Gregory, 1989). Detection selectivity reduces the number of interfering compounds in a chromatogram and is therefore of critical importance in identification and quantification of the folate forms present in the purified extracts (Vahteristo *et al.*, 1996).

Although HPLC holds a high potential for future analysis of folates in food, it has several limitations. One of the major difficulties associated with the HPLC method is the rigorous sample clean-up procedure prior to final injection. Currently, there is a lack of valid purification methods suitable for most of the food matrices which is a hindrance to accurate analysis of individual folates. The applicability of the present purification methods such as affinity chromatography based on folate binding proteins and anion exchange resins to a range of food matrices seems promising but it is yet to be tested and validated with the microbiological assay. Besides the fact that the purification of folate in foods is tedious and being an expensive procedure, it also needs good analytical skills. Although some researchers have reported good agreement between total folate values determined by HPLC and microbiological methods, various studies have reported consistently lower values (less than 50%) for total folate analysed by the HPLC method (Ginting *et al.*, 2002a & b; Kariluoto *et al.*, 2001). The limitation of HPLC detectors to identify some of the folate derivatives and complex sample extraction and purification procedures resulting in the loss of sensitive folate compounds might have caused the lower folate result using the HPLC analysis.

The lack of specificity, along with underestimation, has been an issue with many HPLC methods. HPLC-MS (HPLC-Mass Spectrometry) is now commonly used to detect and quantify the trace level of ambiguous chemicals which is not offered by conventional LC detection methods. However, the use of LC-

MS in analysis of folates in food is almost non-existent. Pawlosky and Flanagan (2001) reported that the precision and accuracy of LC-MS folic acid quantification in a cereal matrix has compared favourably with values obtained through microbiological assay. Further development and validation of LC-MS of folate for specified samples is being paid attention to by the investigators as there is still the possibility of matrix specific problems that must be overcome for different samples. Stokes & Webb (1999) developed a method to analyse the four important forms (5-methyltetrahydrofolic acid, tetrahydrofolic acid and 5- and/or 10-formyltetrahydrofolic acid) of folates in foods using LC-MS.

Bio-specific procedures: Bio-specific procedures or ligand binding methods are sensitive, rapid and specific methods of folate analysis that can be used as an alternative to HPLC and microbiological assays (Finglas & Morgan, 1988 & 1994). They are broadly divided into two groups: firstly, those based on the use of naturally occurring vitamin binding proteins with either radiolabels (radioassay or radio-labelled protein binding assay, RPBA) or enzyme labels (enzyme protein binding assay, EPBA); secondly, those based on the specific interaction of an antibody with its antigen, for example, the radioimmunoassay (RIA) or the enzyme linked immunosorbent assay (ELISA) (Finglas & Morgan, 1994).

The protein binding assays using an enzyme label (EPBA) is a newer method where enzyme-labelled folate-binding protein from cow's milk is used (Finglas, Kwiatkowska, Faulks & Morgan, 1988). Finglas *et al.* (1988) have described the principle of this method which is as follows: the PBA procedure is based on the interaction of folate and enzyme-labelled folate-binding protein (or simply folate receptor, FR). The first step is the immobilization of folic acid to the well surfaces (removable) of microtitration plates. Prepared sample or folic acid standards are mixed with FR/enzyme conjugate in the microwells. During the incubation period, the free folic acid and immobilized folic acid compete for binding with the folate binding protein (FR/enzyme conjugate). The greater the amount of free folic acid in the microwell, the less FR/enzyme conjugate binds to the well surfaces or vice versa. Removal of unbound material by a simple washing procedure allows detection of bound enzymatic activity by addition of a colourless substrate. Bound enzyme conjugate converts the substrate into a blue product; the colour formation is inversely proportional to the free folic acid present in the sample. Quantification of unknown concentrations of folate can be done by comparing the behaviour of standard amount of folate in the system (using regression equation).

Very little research has been performed on the application of a PBA kit method with enzyme labels in foods. Finglas *et al.* (1988) compared folate values of 14 raw and cooked vegetables from EPBA method with microbiological assay which showed a good agreement and linear relationship over the range 0-400 µg per 100 g folate with a correlation coefficient of 0.93. We also validated similar EPBA kit developed by a commercial diagnostic company that used folic acid as the standard for determination of folic acid in fortified foods by the microbiological method. Results from both methods showed no significant difference ($p<0.05$) in the values and showed a high correlation ($r=0.89$, $p<0.001$) (Arcot *et al.*, 2002).

Most radioassay procedures are competitive binding assays, being based on competition between radiolabelled and unlabeled folate compounds for a FBP (Keagy, 1985). Radioassay techniques are widely used for the rapid measurement of plasma and red cell folate in clinical settings (Gregory, 1989). Until now, foods have gained little attention for radioassays largely because of the potential problems related to variation of binding characteristics among the various folate derivatives for FBP (Shane, Tamura & Stokstad, 1980).

Immunoassays are highly sensitive and specific as a result of the interaction of an antibody molecule with its target, a high affinity interaction that occurs even in complex matrices (Finglas & Morgan, 1988). The principle of the immunoassay is almost the same as EPBA except that antibodies are used in place of naturally occurring vitamin-binding proteins (Finglas & Morgan, 1994).

There are still problems to be solved with regards to method performance despite all the efforts that have gone into developing better methods. Many of them are time-consuming and often precision achieved depends on the operation of the skilled personnel. A more rapid and simple method for quantification of folic acid in fortified foods was developed which was an immunoassay performed in an optical biosensor system which utilises the phenomenon of surface plasmon resonance (SPR) as principle (Jonsson *et al.*, 1991; Stenberg, Persson, Roos & Urbaniczky, 1991). This method is rapid and easy to use such as sensitive SPR detection, the specific interaction between analyte and antibody and the low non-specific binding. The biosensor is a continuous flow system with an automatic sample handling and has been validated for the quantification of folic acid in fortified food (Caselunghe & Lindeberg, 2000). The assay itself is completed within 12 hours including sample preparation and measurement and seems to be an attractive alternative to more tedious and less precise assays.

Limitation of biospecific analytical procedures: One of the major limitations of biospecific procedures is its failure to analyse total folate content in foods. Generally these kits have much lower response to folate derivatives other than folic acid, thereby underestimating the natural folate content of foods. It is best suited for analysis of the folate content of fortified foods where folic acid is the major folate derivative. We also observed that the folate values in some fortified foods obtained by the commercial ELISA kit are much higher than that obtained through the microbiological assay. The antiserum obtained by some immunogens e.g., PGA-BSA (bovine serum albumin) on rabbits, exhibits cross-activities to non-folate analogues with folic acid which often leads to over-estimation of folates in the analyte (Finglas & Morgan, 1994). The reagents used in the immunoassay or

protein binding assay kits such as, calibrating standards, buffers, folate binding proteins or antibodies, and colour developing substrates, require proper storage and handling which may otherwise markedly affect the final result. The radioassay procedure that uses radioactivity presents problems associated with its safe handling and disposal. This makes the use of radioassays in routine laboratory folate analysis unattractive.

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An Overview on IPM Approaches for Maintaining Healthy Honeybee Colonies and Quality of Honey in International Market

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This article presents a brief review on IPM approaches for healthy honeybee colony and its Codex, EU and Regional, National standards. Legislation and procedures were reviewed in relation to Nepalese honey export in international market. Monitoring methods of Varroa mite population and using safe-chemicals or other IPM tactics was compared as Nepal follows mainly the Codex standard, compared with other standard in changing context. In IPM measures review on Varroa mite control, a treatment threshold of 117 mites/day (natural fall) and Fluvalinate (Apistan), used as directed on the label, is still be considered the best chemical treatment to ensure colony survival if threshold numbers are exceeded. In the Codex Alimentarius, honey from stingless bees and other bee species is out side the definition of honey. US honey requires the soluble solids not less than 81.4%.The Chinese standards permit up to 24% water whereas in Nepal less than 17% moisture suggested. In EU directive, honey defines as a product obtained from Apis mellifera rather than honeybees. This may be due to illegal restriction on trade of honey. It is found that the presence of antibiotic residue in honey is prohibited in the USA, EU and Japan. In Nepal, it is found that 70% of honey from forest sources including herbs and natural vegetation deserves high quality. The review wishes to explore the relevant information on the development of strategies for healthy colony management on honeybee disease and parasites through IPM measures and facilitate policy formulation in the way of WTO/SPS context to the government and private sectors for honey trade.

Keywords: IPM, Mites, Apistan, Honey standard, Pesticide residue, Codex, EU

Introduction

IPM or Integrated Pest Management is an effective and environmentally sensitive approach to pest management that utilizes a combination of common-sense practices. A goal of IPM is to manage pest populations by keeping their populations below an economic injury level. IPM means not relying on a single pest control scheme. A good IPM program involves selection, integration and implementation of a mixture of biological, cultural and chemical pest control strategies.

IPM is not biological control, although biological control is a useful tactic. IPM is not an organic program although we may integrate organic materials into our control tactic. Nor is IPM anti-pesticide, but generally it attempts to reduce chemical dependency with a mix of control tactics. IPM allows beekeepers to adopt a more balanced approach to mite and disease control that is safer for the beekeeper, bees, hive products and the environment. (Bee Aware, 1999)

Recently *Apis mellifera* beekeeping is intensified in some of the terai district of Nepal. Migration towards the nectar source area for high honey production is practiced by the beekeeper. Honeybees are also using indirectly for crop pollination program. So, the pesticide use in flowering crop as well as inside the bee colony to control disease and pest may cause the honeybee killing and reducing honey quality. Pesticide like organo chlorine, organo phosphorus, carbamates, dinitrophenyl, and some botanicals are observed poisonous to honeybees. Formic acid, sulpher dust and methyl bromide use in various aspect of beekeeping may be found as a reducing honey quality factor. Reducing pesticide residue level in honey is establishing as a pre-requisite component to meet the international standards for honey trade. (Bhandari, 2001). A combination of the stress from mites and disease, along with the low stores of honey because of reduced flower production, may spell trouble. Beekeepers may need to feed sugar syrup to their colonies this winter so the honey bees can survive (Caron, 1999). The moment honey production in Nepal is very low to export in huge amount, i.e., about 864 tonnes. Besides, the honey productivity per hive is very low. Wild honey like *A. laboriosa* fetches much higher prices and sometimes it is said due to presence of narcotic compound. There are 1,25,000 bee colonies domesticated at present, where as the available capacity is one million colonies, i.e., eight times the present capacity. Honey standards related to food safety (GAP, GMP) are compulsory as the basic requirement apart from nutritional or constituent characteristics or other qualities of the products. Honey quality criteria have already been specified in Codex Alimentarious Standards and in the Europian Directives as well. In general the Codex Alimentarious Standards are valid for honey world wide, while other regional standards can also be established under the guidelines of WTO/ SPS agreement. (Summary Report, 2003)

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In Nepal any serious bee disease and pest were not found until 1980, when the serious outbreak of the sac brood disease caused by the Thai sac brood virus (TSBV) occurred first along the eastern border areas sporadic symptoms of iridescent virus infestations are noticed at various places. The Asian mite *Varroa jacobsoni* is long associated with *Apis cerana* but causes no serious damage as it is observed in *A. mellifera*. Occasional visitors also have reported *Tropilaelaps clareae*, *Neocypholaelaps indica* and other predators occur in Nepal. Major bacterial disease EFB and other natural enemies like wasp, harnets, pinemartin are observed as the problems of beekeepers. Annual Technical Report (1999-2000), Entomology division reported that due to continious mite and EFB attack, the performance of colonies from these treatments was very inferior and no honey was produced.

Not so many years ago, raising honey bees was a relatively straightforward task requiring some understanding of bees and management strategies, along with a certain amount of vigilance. Apiary management, and nucleus colony production however, was not the challenge it has become today. The catalyst for the change was the indifferent use of pesticides in flowering crops which kills the honeybees and the raising resistence with antibiotic and miticides inside the colonies. Knowledge and skill to manage the honeybee diseases, mites and judicial use of chemicals and safe alternatives to control them for quality honey production is considered as the technical problem of beekeepers in rural area of Nepal. Honey bee quality analysis (quantitative) and residue control within the set MRL is also one of the major issue in relation to WTO context for marketing of honey abroad.

The demand of Nepali honey is increasing in international market but due to lack of European Union Standard Residues Monitoring Regulation the export of Nepali honey to European market has been forbidden. They put forward different conditions such as standardization of quality control measures, provision of European standard certification technology and enhancement of competitiveness of honey producers.

The main objectives of the review are:

- To familiar with IPM measures for mite control in honeybee colonies
- To know the honey quality parameter and pesticide residue for honey trade in international market
- To find out the misunderstanding of requirements in honey standard among different countries.

IPM Measures

The success of an IPM program hinges on good monitoring of pest levels. If we are to reduce our chemical dependency for bee mite control, survey methods must be developed that allow us to determine the proper threshold levels on which to base control decisions. The ether roll method is not reliable as all the mites in our sample of adult bees, which can vary from as few as 100 to more than 500 bees, do not show up on the glass container. Washing the sample with alcohol or soapy water and then filtering through two meshes to trap mites is time consuming but a bit more reliable. Opening and examining drone brood (pupae) cells, like the ether roll technique, tells us if mites are present but we do know what numbers should indicate the need for control. All these methods are destructive resulting in dead bees/brood.

MAAREC research programs are focusing on sticky boards as a more reliable method of monitoring populations of Varroa mites. A Georgia/S. Carolina study reported a treatment threshold of 117 mites/day (natural fall) using sticky boards to monitor mite fall. MAAREC studies (at Delaware) reveal 40 mites/day of natural mite fall might be a better threshold basis.

Once threshold levels have been exceeded, IPM measures should be taken to lower numbers below that injury level. Pesticides can do this rapidly. One IPM strategy is to utilize pesticides with more specificity and lower toxicity. Fluvalinate (Apistan) is such a chemical relative to Coumaphos. Although resistance is present, and spreading, Apistan, used as directed on the label, should still be considered the best chemical treatment to ensure colony survival if threshold numbers are exceeded. Another chemical, formic acid gel, is nearing registration and it should prove useful for bee mite control. A number of essential oils (biopecticides) have been tested by Penn State, the MAAREC project and by other researchers such as Jim Amrine of West Virginia. Several have been found that may be effective but delivery and dosage levels have yet to be determined. Use of other techniques might help keep mite levels from reaching injury (threshold) levels. The sticky board technique, useful to monitor mite numbers, may also be a means of reducing bee mite numbers when sticky boards are used continuously. Modification of the bottom board may also be a means of reducing mite numbers to reduce dependency on chemical pesticides. A promising area of study points to management of bee colonies that involve removal of drone brood or an interruption in the brood cycle via caging of the queen. One variation is to place all colony brood in a select few colonies for treatment with Apistan and then redistribution to colonies. This limits the number of colonies exposed to the pesticide.

Drone brood trapping is an IPM technique that shows some promise though it is labor-intensive. This technique requires that brood in bee colonies be removed and only combs with drone brood cells used for a period of two weeks. Mites invade preferred drone brood cells during this broodless period. The drone brood combs are removed at the end of the period and put in a freezer to kill all mites. Another useful IPM technique is to use bee stock resistant to or tolerant of Varroa mites. Some larger beekeepers have been selecting for colonies with fewer mites using only natural selection. Working with a commercial beekeeper, colony populations of Varroa mites initially at 120 mites/100 bees have decreased to 6 mites/100 bees in the Tucson project. One problem is the bee stock is at least partially Africanized, so exporting these to other parts of the country seems unlikely. Hygienic bee populations that are more diligent house cleaners may also be useful stock. Spot pesticide treatments, only when and where mite population exceed threshold numbers, and vigorous use of the entire arsenal of control tactics in an integrated pest management approach will best serve beekeepers, our bees, and our clientele in the long run.

The Codex Alimentarius Worldwide Standard

The standard is divided into two parts. Part One covers all honey produced by honey bees which is processed and ultimately intended for direct consumption. Part Two is intended to cover honey for industrial uses or as an ingredient or in bulk. This Part stiil has to be developed.
Note that honey from stingless bees and other bee species is outside the definition and therefore can no longer be called honey. Thus there must be a Part Three to the standard but the compositions of stingless bee honeys are not well characterised, making the development of the standard difficult.

Regional and National Standards

The United States is anomalous in having only agricultural grades for honey but the National Honey Board is seeking a Standard of Identity for honey. The grades characterise by colour, flavour, clarity and the absence of defects and require the soluble solids, as determined by refractometer to be no less than 81.4%.

Mexico developed APNMX-F-036-NORMEX-2003 last year. This has been changed to bring it into line with international norms as it permits very high levels of very toxic pesticides as well as veterinary drug residues.

Table 1: The compositional standards of Honey

Parameters	Part I Honey	Part II Honey
Fructose + glucose	Blossom honey, not less than 60% by weight	Honeydew honey and blends of honeydew honey and blossom honey, not less than 45% by weight
Moisture content	In general, not more than 20%	Heather honey (*Calluna*), not more than 23%
Sucrose content	In general, not more than 5%	*Medicago sativa, Citrus, Robinia pseudoacacia, Hedysarum, Banksia menziesii, Eucalyptus camaldulensis, Eucryphia lucida* and *Eucryphia milliganii* honeys, not more than 10%
		Lavandula and *Borago officinalis* honeys, not more than 15%.
Water-insoluble solids content	In general. not more than 0.1%	Pressed honey, not more than 0.5%
Electrical conductivity	Honeys not listed under b) or c) and blends of these honeys, not more than 0.8 mS/cm	b) Honeydew and chestnut honey and blends of these except with those listed under c), not less than 0.8 mS/cm.
		c) Exceptions are *Arbutus unedo, Erica, Eucalyptus, Tilia, Calluna vulgaris, Leptospermum and Melaleuca*
Free acid	Not more than 50 milli-equivalents of acid per KG	
Diastase determined after processing and blending	Not less than 8 Schade units.	Not less than 3 Schade units for honeys with a low natural enzyme content, e.g.*Citrus.*
Hydroxymethylfur-fural determined after processing and blending.	Not more than 40 mg/kg (subject to the provision under diastase)	Honeys of declared origin from regions with tropical ambient temperatures and blends of these honeys, not more than 80 mg/kg

The Chinese standard allows 'superior product' and 'acceptable product', the latter permitting up to 24% water. Honey containing so much water is sure to ferment. Such a double standard seems a most unfortunate idea.

The EU Directive basically follows the Codex standard but includes requirements for baker's honey and makes the limits for acidity, diastase, hydroxymethylfurfural and electrical conductivity mandatory. But there is a great deal of other EU legislation that impacts on honey and that is considered below.

Antibiotic Residues

A major quality issue in recent years has been the presence of antibiotic residues in honey. It may be worthwhile to reiterate the facts as there often seem to be misunderstandings about the requirements in different countries. The presence of antibiotic

residues in honey is prohibited in the USA, EU and Japan. The very similar position in the USA and in the EU is shown in Table 2. (Julia Pirnack of the US National Honey Board for making the US position clear).

The EU Monitoring Programme

Directive 96/23/EC is on measures to monitor certain substances and residues thereof in live animals and animal products. This has been brought into national law, for example, in UK as The Animals and Animal Products (Examination for Residues and Maximum Residue Limits) Regulations 1997, as amended.

Member states have been required to carry out a monitoring programme for veterinary drugs since 1.1.99 on honey produced within the member state. There is no mandatory requirement to analyse imports which is why the importing country must do it in an approved way. Don't imagine this is a soft option, as importers and packers check every consignment for residues because they do not want to have the embarrassing and costly process of a product withdrawal once the honey is in the shops. Article 29 of Directive 96/23/EC states 'Inclusion and retention on the lists of third countries provided for in Community legislation from which member states are authorized to import animals and animal products covered by this Directive shall be subject to submission by the third country concerned of a **plan setting out the guarantees** which it offers as regards the monitoring of the groups of residues....' 'Where the requirements [above] are not complied with, inclusion of a third country on the lists of third countries laid down by Community legislation....may be suspended.' This has happened in the case of China.

Table 2: Position of Antibiotic residue in honey in USA and EU

Veterinary Drug	*Maximum Residue Limit*	
Antibiotics	**USA**	**EU**
Oxytetracycline, use permitted	Zero	Zero
Fumagillin, use permitted	Zero	Zero
Tylosin, under evaluation in US	Zero	Zero
Lincomycin, under evaluation in US	Zero	Zero
Streptomycin	Prohibited	Prohibited
All other antibiotics	Prohibited	Prohibited
Other Veterinary Drugs		
Sulphonamides	Prohibited	Prohibited
Fluvalinate (Apistan)	50 µg/kg	None considered necessary, NCN
Flumethrin	Not permitted	NCN
Amitraz (Tactic, Apivarol)	1000 µg/kg	200 µg/kg
Cymiazole (Apitol)	Not permitted	Permission expired
Formic acid	Exempt	NCN
Lactic acid	Not permitted	NCN
Menthol	Exempt	NCN
Thymol	Exempt	NCN, but may be deleted
Mixed oils (i.e. Apilife VAR)	Exempt	NCN
Coumaphos (Perizin)	100 µg/kg	100 µg/kg
Oxalic acid	Not permitted	NCN

The EU Cascade Procedure

This procedure is used when there is no authorised product for a condition in a particular species. If a drug is licensed for use in another food-producing animal, or for another use in the same animal, a veterinarian can prescribe the drug for a small number of animals on an ad hoc basis. For example, in UK there is no product licensed to treat European Foulbrood, so it is legal for a veterinarian to prescribe the administration of tetracyclines to a hive of bees or a number of hives in the same place by way of treatment. A similar procedure in a third country would be regarded as reasonable, but care must be taken that any residues in honey are below the LOQ - level of quantification. It would be risky for a third country to adopt this approach because importers are insisting on compliance with EU without reference to Cascade. Using the Cascade principle could result in very long delays before importation of a consignment was accepted as legal.

Action Procedures in the EU

If the Department of the Environment, Food and Rural Affairs in UK or its counterpart in another member state finds levels above the Limit of Quantitation (LOQ) of any drug for which there is no MRL or above the MRL where that has been set, the finding is reported to the Chief Veterinarian. He would expect to discuss this with his counterpart in the country of origin of the product and obtain an explanation.

In UK it has been decided to regard 50 ppb as an Action Level whereas Germany, for example, uses the Limit of Quantitation

of the method of analysis, often 10 ppb. The industry is asking for these Action Levels to be harmonised as the present anomalous situation can cause honey rejected in Germany, for example, to be 'dumped' in UK. The EU is now working on new requirements and discussing the concept of a Minimum Residue Level.

If a third country had a licensing system and a procedure for setting MRLs, then it would be able to argue for the presence of residues of approved drugs up to the MRL. Then either at national government level or Commission level, a decision would be made to permit or prohibit product containing the residue.

EU Legislation on Pesticide Residues

There is no EC legislation relating to pesticide residue limits in honey but countries such as Germany, The Netherlands and Italy do have limits. In some cases these are specific to particular substances and in other cases there is a general limit of 10 µg/kg or 50 µg/kg. In general, pesticide residues in honey have not been a significant problem. Deaths of hives from crop spraying are a hazard to beekeepers but this does not generally result in high levels of residues in the honey. Consumers in the European Union are extremely sensitive to the issue of pesticide residues in food and it is particularly important that a product such as honey that is regarded as natural and pure should be as free from residues as possible. Perhaps unfortunately, methods of analysis are also exquisitely sensitive and vanishingly small levels of pesticides such as lindane are occasionally found.

Table 3: Honey composition standard for Nepal

Parameters	Standard
Moisture	23% Max
Ash	0.5% Max
Sucrose	Pure Floral Nectar Honey 5% Max
	Other honey 10% Max
Reducing Sugar	Pure Floral Nectar Honey 65% Min
	Other honey 60% Min
Fructose Glucose Ratio	0.95 Min
Acidity as Formic Acid	0.2% Max
Water insoluble solid	0.5% Max
Hydroxymethyl furfural	40 mg/kg Max

[Source: Nepal Gazette, 2001]

Comparison of Nepal honey standard with codex part I honey shows that moisture percentage, water insoluble solid percentage and reducing sugar parameters are not in the same standard, although, the general standard of Nepal honey must be revised so as to meet codex and EU standard applying with appropriate analysis method.

Honey quality includes quality in terms of food quality and standards of processed honey produced by manufacturing companies like Dabur. Incase of honey export, Nepal used to export honey to Norway but the adoption of council Directives96/23/EC by EU as well as Norway through EEA Agreement necessitated Nepal as a trading partner to present a residue control program. Nepal failed to present such a program required by council Directive 96/23 and so was deprived of the opportunity to appear in the list of countries eligible to export animal products including honey to EU.

In Nepal, the present legislation provides the responsibilities for inspection and control to the authority but implementation part is not strong. Nepal honey is considered high quality as 70% of total from diversified natural flora and forest but the monitoring, validation and certification system should be established. The food safety, hygiene and processing standard should be developed.

In Nepal, system on various aspect of pesticide residue and quality analysis of honey has not been yet advanced. Although the work based on findings of research and international standards is being improved in the context of WTO/SPS agreement. Beekeepers are getting technical services to honey quality (mostly qualitative test) like moisture content by refractrometry test, hydroxylmethyl furfural (HMF) content by titration method, the apparent reducing sugars and apparent sucrose content are measured by the Fehling method. But water insoluble solids content, proline (a.a) content, specific rotation of honey, diastase by schade method invertase activity by invertase number (IN). The IN indicated the amount of sucrose per gram hydrolised in one hour by the enzymes contained in

Coumaphos is essentially a pesticide but you will have noted above that it is also used as an acaricide. This may make it difficult for legislators to decide if they are dealing with a medicinal product residue or a pesticide residue or both, with consequent confusion in the legal status of the product.

In general, if pesticides are used, for example against wax moth or other infestations, it is important to remember the need to ensure negligible levels of residue in the honey. This may require expert advice from bee inspectors and apicultural advisers so that the best product is chosen and used correctly.

The industry can be so concerned about pesticide residues, even in member states that have no specific national legislation that they will include requirements in their contracts, for example, specifying compliance with the residue limits stipulated in the European Pharmacopoeia.

Quality Status of Honey in Nepal

Nepal basically follows the Codex standard for honey and not yet developed the program on pesticide and antibiotic residue in honey. Although the work is initiated (sampling, testing honey with appropriate method and Plan setting for guarantee) in the same line with co-ordination of Department of Food Technology and Quality Control, Department of Agriculture, Nepal Standard, Bee Development Section, and private honey traders. Department of Food technology and quality control has developed the honey standards (Table 3).

100 gm of honey under test condition, are required parameters should be established before it can be accepted internationally.

Saville, Naomi, Shrestha, MahaLaxmi, Acharya, & Joshi reported the Impact of honey harvesting and processing methods on honey quality in West Nepal. It is found that out of different categories of honey, Apis cerana cerana fresh honey from slightly improved harvesting & processing consists better honey standard in comparison with other categories of honey. Thirty-three A. cerana cerana honey samples were analysed (Table 4) in west Nepal (Humla, Jumla).

Table 4: Results of analysis of Apis cerana cerana honey

Categories of honey separated by processing method	*Statistic*	*Water %*	*Peroxide activity µg/g/hr*	*HMF mg/Kg*	*pH*	*Reducing sugar % Glucose*	*Sucrose %*	*Electrical conductiv-ity US/cm*	*Ranking of plant families or genera by frequency of pollen grains in 18 of the Apis cerana honey samples*
Traditionally harvested & fresh Apis cerana cerana honey	Mean	18.86	17.50	< 40	4.61	70.78	3.48	33.16	1st : Compositae: (including Azaractum-, Aster-, Ageratum-, Taraxacum- and Cirsium-, types) 2nd : Labiatae, Rosaceae (incl. Malus sp.), 3rd : Zygophyllaceae, 4th : Fagopyrum sp., 5th : Poaceae, 6th : Balsaminaceae, 7th : Brassica, 8th : Curcurbitaceae, 9th : Rumex, 10th : Umbelliferae, Alnus, Loranthus, Citrus, 11th : Polygonaceae, Salix, Malvaceae, Amaranthus, Mimosaceae, PinusStarch cells found in some samples. One sample contained honeydew.
	n	18	18	18	18	18	18	11	
	SE mean	0.44	2.16		0.10	0.88	0.38	12.44	
Traditionally harvested & stored Apis cerana cerana honey	Mean	18.60	9.50	< 40	4.68	69.92	1.72	11.50	
	n	5	5	5	5	5	5	1	
	SE mean	0.78	4.87		0.15	1.86	0.24		
Cooked Apis cerana cerana honey	Mean	21.30	8.00	< 40	4.58	69.38	3.26	14.05	
	n	5	5	5	5	5	5	4	
	SE mean	2.08	5.11		0.13	2.10	1.14	1.31	
Apis cerana cerana fresh honey from slightly improved harvesting & processing	Mean	19.36	20.50	< 40	4.62	70.79	3.10	16.60	
	n	5	5	5	5	5	5	4	
	SE mean	1.10	5.03		0.24	2.40	0.67	4.22	

Conclusions

In order to empower the beekeepers with healthy honeybee colony and quality honey production, IPM measures based on understanding bee ecosystem, can be used through beekeeping farmers field school. Most possibly this approach is a new concept with systematic order for reducing residue of bee products in Nepal. Special topics can be included are study on mite species, appropriate queen rearing method, bee flora, honey harvesting method, honey processing, and residue analysis using accepted methods.

Selection of Disease free colony through, sanitation of bee equipments as the major source of disease contamination and colony with low frequency of swarming tendency, selecting colony with low frequency of absconding tendency, having good superseded tendency of appropriate condition, healthy queen introduction through appropriate queen rearing method, concentric egg laying pattern and good fecundity of queen can be practiced for healthy bee colony maintenance.

Study of mite control through identification of mite and their lifecycle, break brood cycle by queen caging, using sticky board to count fallen mite, drone cell capturing and counting, use essential oil and Fluvalinate after exceeding injury level, feeding sugar syrup for colony strength is recommended. Honey Quality response to different management practices like No use of antibiotics and miticides, harvesting capped or ripe honey, using honey extractor and glass bottle for packing, testing major quality parameters and pesticides residue. In order to produce and develop healthy apiary and quality honey with technical justification, we need to provide good beekeeping practices and good honey processing services (according to GAP and GMP guidelines) to the beekeepers. Better to focus on programmes like beekeeping friendly pesticide use, various action researches

in beekeeping, establish IPM benefits with beekeepers, building field staff, women beekeepers & IPM, IPM beekeeper's field school, training to the trainers, quantitative test of honey, quality honey production and marketing based on the scientific study is suggested.

Regarding the honey quality for export in international market, the present legislation, rules and laws related to food quality are under amendment in Nepal. The monitoring, validation and certification system of honey should be established in the line of codex alimentarius and other regional/ national standards under WTO/SPS agreement. A national honey board is suggested to seek a standard of identity for honey focused with Apis cerana and other bee honey in Nepal.

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Calcium Carbide for Artificial Ripening of Fruits-Its Application and Hazards

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This article presents a brief review on artificial ripening of calcium carbide with especial reference to its application and related health hazards. Various reports suggest that the use of calcium carbide as a ripening agent is better to be strictly monitored and controlled.

Keywords: Acytelene; Artificial ripening; Calcium carbide; Climateric and non climateric fruits; Ethylene

Introduction

Nepal has its huge potential for the production of different agricultural produces such as cereal grains including millets, legumes, fruits and vegetables, spices & condiments, among others due to its varying topography and accordingly different climatic conditions. The location of Nepal favours in producing many of the agricultural produces that are produced in all, tropical, subtropical and temperate types of climate.

As emphasised by the Agricultural Perspective Plan (APP) sufficient thrust was given for the production of High Value Crops (HVC) including fruit and vegetable products in the 9th five-year plan. Similarly, emphasis has been given in the 10th plan for the production of horticultural products. The latest statistics of Nepal shows that 511,397 MT of fruits and 1,890,100 MT of vegetables were produced in the country in the fiscal year 2003/04.The production figures of different types of fruits for the year 2003/04 is as given in the Table 1 below:

Table 1: Production of fruits for 2003/04

Fruits	Production (MT)
Citrus	148,010
Winter (Deciduous)	94,988
Summer (Tropical)	268,399

(Source: MOAC, 2003/2004)

Citrus fruits of importance that constitute orange, sweet orange, lime, lemon and others. Similarly, winter (deciduous) fruits comprises mainly of apple, pear, walnut, peach, plum, apricot, persimmon, pomegranate, and others. In the same way, in the order of highest production figures, tropical (summer) fruits are mango, banana, guava, litchi papaya, areca nut, jackfruit, pineapple and coconut.

Table 1 indicate that fruit production of the country has sufficient role in contributing to the national Gross Domestic Product (GDP). Therefore, its production, handling and marketing i.e. from the farm to table continuum, is very important. Some fruits are harvested only after they are ripened in the orchard and some can be harvested after they are fully matured before ripening, to facilitate for the handling and marketing of such products. The latter types of fruits before placing on the market are to be ripened. For this, artificial ripening, especially by the use of calcium carbide is being practiced in Nepal.

To this end, different articles have been published in different media for and against the use of such chemicals and have created some confusion among the consumers. Therefore, this article attempts to inform the consumers whether the use of such chemical is harmful or not, how to use such ripened fruits without violating the safety norms and how to identify such fruits on the market.

Role of Ethylene in Ripening

The internal ethylene concentration of climacteric fruits varies widely, but that of non-climacteric fruits changes little during development and ripening (Burg and Burg, 1962). Ethylene, applied at a concentration as low as 0.1 - 1.0 micro litres per litre for one day, is normally sufficient to hasten full ripening of climacteric fruits, but the magnitude of climacteric is relatively independent of the concentration of applied ethylene. In contrast, applied ethylene merely increases the respiration of non-climacteric fruits, the magnitude of the increase being dependent on the concentration of ethylene. Moreover, the rise in respiration in response to ethylene may occur more than once in non-climacteric fruits in contrast to the single respiration increase in climacterics (Baile, 1964).

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Table 2: Internal ethylene concentrations in fruits

Fruits	Ethylene (µl/L)
Climacteric	
Apple	25-2500
Pear	80
Peach	0.9-20.7
Avocado	28.9-74.2
Banana	0.05-2.1
Tomato	3.6-29.8
Non-climacteric	
Lemon	0.11-0.17
Lime	0.30-1.96
Orange	0.13-0.32
Pineapple	0.16-0.40

(Source: Burg & Burg, 1962)

Artificial Ripening

Artificial ripening is done to achieve faster and more uniform ripening. It is the process by which ripening is controlled and product may be achieved as per requirement by controlling the different parameters. Climacteric fruits, particularly tropical and subtropical species, are frequently harvested when less than fully ripe and then transported, often over considerable distances, to areas of consumption. Here these fruits are ripened to optimum quality under controlled conditions of temperature, relative humidity, and with some fruits, through the addition of ripening gases. A further advantage of controlled ripening is to improve uniformity of ripening of fruit. The use of relatively high ripening temperature may also minimize the development of rots in ripe tropical fruits. In contrast, non-climacteric fruits generally undergo little or no desirable change in composition after harvest and must not be harvested until fit for consumption.

A significant proportion of the world production of bananas of approximately 18 million tonnes is ripened under controlled conditions. The banana is unusual in that it can be picked over a wide range of physiological ages from half grown to fully-grown and ripened to excellent quality with the aid of ethylene. Acetylene, generated by adding water to calcium carbide, produces a ripening response, but in practice at least a concentration 100 times higher is required (Wills *et al.*, 1989). The concentrations of ethylene required for the ripening of various commodities vary but in most cases are in the range of 0.1 to 1 ppm. The time of exposure to initiate full ripening may vary, but for climacteric fruits exposure of 12 hours or more are usually sufficient. Full ripening may take several days after the ethylene treatment.

In general optimum ripening conditions are given in Table 3.

Table 3: Optimum ripening conditions for different fruits

Parameters	Value
Temperature	18 to 25°C
Relative humidity	90 to 95 %
Ethylene concentration	10 to 100 ppm
Duration of treatment	24 to 72 hours depending on fruit king and maturity stage
Air circulation	Sufficient to ensure uniform distribution of ethylene, which reduces the effectiveness of ethylene

(Source: Reid, 1992)

Sources of Ethylene

Explosion proof ethylene mixture: - 6% ethylene in carbon dioxide by weight

Ethylene generator: - ethanol is heated in the presence of catalyst to produce ethylene.

$$C_2H_5OH \longrightarrow C_2H_4+H_2O$$

Ethephon: - Chemical name- 2-chloroethane phosphonic acid, commercial name- Ethrel, Florel, Cepa

It is acidic in water solution and above pH 5 it liberates ethylene.

Use of ripe fruit: - Ripe fruits with ethylene production can be used to ripen or degree on other fruits.

Calcium Carbide: - When hydrolyzed, it produces acetylene, containing trace amounts of ethylene that are sufficient to be used in fruit repining. Acetylene, the end product of Calcium Carbide and Water provokes the same effects as the fito-hormone ethylene, but neither Calcium Carbide nor synthetic ethylene when used to "ripen" less mature fruit, produce results that approach those of fruit picked closer to it's peak.

$$CaC_2 + H_2O \longrightarrow CaCO_3 + C_2H_2$$

Comparative effectiveness of ethylene and related compounds are given in Table 4.

Table 4: Comparative effectiveness of ethylene and related compounds

Compound	Relative Activity (moles/unit)
Ethylene	1
Propylene	130
Vinyl chloride	2,370
Carbon monoxide	2,900
Acetylene	12,500
1- butene	140,000

(Source: Burg & Burg, 1966)

Use of Calcium Carbide and its implications

Identification: Calcium carbide is a greyish black, lump shaped solid or crystal (sugar or sand like), which has slight garlic like odour. It is readily produced by heating calcium oxide with charcoal under reducing conditions. It is commonly used to make acetylene and other chemicals and in metallurgy. It is considered as cheap source of ripening agents and also easy method to use by general people, but they are not conscious about the hazard that it can commence.

Hazard Summary: Calcium Carbide is extremely hazardous compound. The health hazard aspect of calcium carbide can be described on the following way:

Acute health effects: The following acute (short term) health effect may occur immediately or shortly after exposure to calcium carbide: -

- Contact can severely irritate or burn the eyes and skin causing permanent eye damage and ulcers on skin.
- Exposure can severely irritate the mouth, nose and throat causing sores cough and wheezing.
- Irritate the lungs causing coughing and/or shortness of breadth. Higher exposure may cause a build up of fluid in the lungs (pulmonary oedema), a medical emergency, with severe shortness of breath.

Calcium carbide contains traces of Arsenic and Phosphorus. Early symptoms of Arsenic or phosphorus poisoning include vomiting, diarrhoea with or without blood, burning sensation of the chest and abdomen, thirst, weakness and difficulty in swallowing and speech and garlic like odour of breath. Other effects include numbness in the legs and hands, general weakness, cold and damp skin and low blood pressure. While most cases of arsenic and phosphorus poisoning are detected before they become fatal. If not treated in time, these can prove fatal. "Pregnant women are particularly vulnerable. The chemical residue in the fruit could lead to miscarriage."

Chronic Health effect: The chronic (long term) health effect can occur at some time after exposure to calcium carbide and can last for months or years. Calcium carbide has not been tested for its ability to cause cancer and reproductive hazard but may cause bronchitis to develop with cough, phlegm, and /or shortness of breath (HSFS, 2003).

Safety aspects in handling Carbide

The hazardous aspect of calcium carbide is in both inhalation and intake of residue but acetylene gas seems to be in practice and does not indicate the negative impact on the fruit intake. Even literatures show that calcium carbide is still being practiced outside. The exposure hazards of carbide may be controlled by using prescribed personal protective equipments, following standard procedures of workplace control, practices, handling and storage. The major hazard from the consumption of calcium carbide treated fruit is the residual effect, so using the carbide in closed and separate atmosphere to release acetylene and use the gas in separate ripening room seems to be safe. Literatures show that carbide wrapped in newspaper can be used as the generator in separate section. Water vapour from the fruit releases sufficient amount of gases from carbide to cause ripening. Some literatures report that there is a practice of using about 100gm of carbide per 50 kg of fruit.

Food regulation on the use of calcium carbide

Considering the possibilities of aforementioned hazardous effects, the use of carbide has been banned in many countries of the world. Rule no. 44-AA of the Prevention of Food Adulteration Act (PFA India) and subsequent rules have strongly prohibited the use of carbide gas in ripening of fruits expressing that " No person shall sell or offer or expose for sale or have in his premises for the purpose of sale under any description, fruit which have been artificially ripened by use of acetylene gas, commonly known as carbide gas". Similarly, Part 7, Rule no 19 (d) of Nepal Food Regulation 2027 has strongly prohibited the use of carbide gas in ripening of fruits. Thus it is advised not to use such chemicals so as to ripen the fruits.

Detection of artificially ripened fruits

Although the use of carbide has been banned under the food act and is punishable, but a few seems to care. Artificially ripened fruits are much likely to occur on market. It is suggested that naturally ripened fruits are not uniformly yellow. Artificially ripened fruits bear white streaks on the skin, whereas naturally ripened fruits would be mix of green and yellow. Close scrutiny can make it possible to identify fruits that have been treated with carbide. When tomatoes are uniformly red, or mangoes and papayas are uniformly orange, one could easily make out that carbide may have been used. Banana can also be identified if the stem is dark green while the fruits are all yellow. Suspected sample may be tested for the phosphorus and arsenic residue on the surface of the fruits on the lab.

Conclusions

Fruits and fruit vegetable such as tomatoes are being treated with calcium carbide to ripen them fast. The use of carbide, considering its hazardous aspects, lack of knowledge about its standard procedures for safety use among concerning people and being banned by the regulation, is to be strictly monitored and controlled. While purchasing fruits and fruit vegetables, that are prone to artificial ripening in the Nepalese food chain, it is advised not to select homogenously ripened and eye catching bright coloured fruits. In case of banana if the stems are green and the fruits are well ripened then it could be suspected of the use of carbide.

Washing and peeling operations before eating a fruit, in case of suspicion, could help in minimising the risks associated with the use of carbide.

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Fermented Foods of Nepal-A Brief Review

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The most popular fermented foods of Nepal are Yak cheese, Kinema, Gundruk, Sinki, Mesu, Dahi, Mahi, Chhurpi, Selroti, Jandh, Rakshi and Achar. This article presents a brief review on the preparation and consumption pattern and cultural and nutritional importance of those food products of Nepal.

Keywords: *Kinema, Gundruk, Sinki, Mesu, Selroti, Jand, Rakshi, Chhurpi*

Introduction

Fermented foods have been part of staple foods in the mountainous region of Nepal and Bhutan, as well as for the Nepalese origin ethnic community living in different parts of the India including Sikkim, West Bengal Hills, Dehradun, Assam, Arunachal Pradesh, Manipur, Meghalaya including Hong Kong and so on. These foods are important component of diet for these people as staples, adjunct to staples, condiments and beverages. A variety of fermented foods like *Kinema, Gundruk, Sinki, Mesu, Jandh, Chhurpi, Shelroti* etc. are prepared in family scale. It is a practice protected by ethnic and family tradition. The preparation methods are traditional, and needs to be studied, developed, scientifically and commercially standardized for the cottage as well as the industrial production and uses. These foods are highly nutritious, easily digestible, tasteful and sensorily acceptable.

As is evident, fermentation has been used to preserve the foods for long-term use or prolong the shelf life of foods. The most popular fermented foods are *Kinema* (fermented soybean), *Gundruk* (*Brassica campestris* leaves), *Sinki* (*Raphanus sativus*), *Tama* (succulent bamboo shoot), *Dahi* (similar to yoghurt), *Mohi* (buttermilk), *Sher*, *Shergum* (soft cheese from buttermilk), *Chhurpi* (dried very hard cheese from buttermilk), *Selroti* (deep-fried preparation from rice flour), *Jand* (local beer from rice/maize), *Rakshi* (alcohol distilled from fermented rice, maize, millet), *Tumba* (fermented millet drink), and varieties of fermented acidic pickles *(Achar)*. *Murcha* (*Saccharomyces* sp.) is the starter for the alcoholic beverages and *Bacillus subtilis* for making *Kinema*. *Gundruk, Sinki, Tama* and varieties of *Achar* (pickles) are some of the popular fermented vegetables from the hills and mountains of Nepal and Bhutan. *Gundruk* is very indigenous to mountains, and is recognized as a symbol of identity of Nepalese people. These fermented foods are mostly prepared for family consumption, as well as for the souvenir or gifts while visiting relatives and friends. However, brilliant prospects of initiating small-scale income generating enterprises for women exist, which could help in rural poverty alleviation.

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Fermented milk products are other popular food products in this mountainous region. In Nepal, around 85 % of 1.1 million metric ton milk produced per annum is processed at the household level producing traditional and very popular fermented milk foods are *Dahi, Mahi, Chhurpi*, Yak cheese and other cheeses. These are used as staple food and consumed with rice, maize meal and bread. Nepal produces around 200 metric tones of yak cheese annually, which is very typical to Nepal, and the production started some five decades before. This paper briefs major fermented foods produced and consumed in Nepal.

Yak Cheese

Yak Cheese is considered as a Souvenir of Nepal in the world. For centuries, Yak milk is traditionally processed into indigenous dairy products like fermented milk, *Nahuni* (local butter) *Ghee*, *Chhurpi* and so on in the high Himalayan region. These products were traditionally traded with Tibet. In order to economically utilise this surplus milk and provide an alternate market to the producers, His Majesty's Government of Nepal established the first cheese plant in Langtang (3000-3400 metres above sea level) in 1952 with FAO technical assistance.

Yak milk products are important source of nutrition as well as income to the local yak and *Chauri* herders in the high alpine region. Nepal is one of the first countries in Asia to establish yak cheese industry in the early fifties (1952). Cheese is produced by both private firms (20) and government parastatal (6) to the tune of around 100 metric tons annually (Thapa, 2000). The small-scale yak cheese technology is similar to the one used in the production of Swiss Gruyere type hard cheese in Switzerland. Yak cheese is excellent high value product from milk. Approximately 1-kg cheese and 0.43 kg butter is produced from 10 litres of yak milk. Beside others, poor technical know how and skill in cheese making is one of the major constraints of yak cheese industry, which are located at remote high altitude locations. Government commitment with a proper focussed policy can help to strengthen the yak milk based industry. In the present context, the member countries could be benefited with Nepal's experience in yak cheese production and marketing. An Asia Regional Yak Cheese Training Centre would be useful in disseminating and

transferring the Nepal's five-decade experience of producing yak cheese on most adverse conditions.

The yak cheese industry is of significant importance form rural income and employment generation points of view in the area of production. More than four thousands people are estimated to be earning their livelihood form the yak cheese sub-sector. The estimated industry revenue and foreign exchange earning for 1994 are NRs (71NRs=1US$) 31,981,175 and US$ 494,000. If Nepal is to promote agro-industries based on livestock products, yak cheese is one of the ideal products. It is a high value-high demand dairy product with established internal as well as export markets potentials (Colavito, 1994).

Yak milk differs from cow milk in composition, fat ranges between 7-9%, protein 5-7% and TS 17-19%. Thapa & Sherpa (1994) reported the yak cheese making technology adopted by the Nepalese yak cheese producers in the 24th International Dairy Congress held in Melbourne, Australia. Again, Thapa (1997) described further improved yak cheese making procedures used by the yak cheese manufacturers in the 2nd International Congress on yak held in Xining, China. Most recently improved and standardised method is presented below (Thapa, 2000)

Raw milk is standardized to 3.5% fat. Can pasteurization of cheese milk at 65°C/5 minutes is carried out by immersing in a boiling water bath and cool in a water-trough to 33-35 °C. Cheese milk is transferred to 200-300 litres copper kettle and put on a traditional fireplace. 0.5% culture (*Streptococcus thermophilus* and *Lactobacilus helveticus* 1:1) is added. After 5 minutes, rennet solution (2.5 g dissolved in 500 ml boiled and cooled water for 100 litres of milk) is added and stirred for one minute before allowing to set (33°C) by covering the kettle. Top curd is turned using scoop after 30 min. and allowed setting for 5 minutes. The curd is cut and after 5 min stirring started very slowly. Stirring is continued for 25 min (32°C) and allowed to settle for 5 min. The curd is cooked to 50 -53°C in 30 minutes. The temperature is gradually raised in the following manner; 30 to 35 °C in first 10 minutes; 35 to 45° C in second 10 minutes; 45 to 50 °C in last 10 minutes. After the temperature reaches 50° C, Stirring is continued for 25-30 minutes until the curd is ready for moulding and pressing. Then the curd is fished out in the cheesecloth after the cheese maker feels the curd is sufficiently firm or cooked. The curd is put in the moulds and pressed using stone slabs.

The blocks are turned after 5 minutes, 15 minutes, 45 minutes and after 1.5 hours. Wet cloth is replaced by dry cloth after 1.5 hours. Fifth turn is given after 3.5 hour and dry cloth is used. After six hours, cheese is pressed without cloth till next morning. Total pressing time ranges between 16-17 hours (Adhikari, Bajracharya & Thapa, 2001).

Cheese blocks are brined in a saturated salt solution (24%) generally for 48 h, but the brining time is proportional to the weight of the blocks. 5-6 kg blocks are brined for 36 h whereas 14-15 kg blocks are brined for 72 hours.

The cheese blocks are stored for ripening at 10-15°C and 85-90 % humidity for first two weeks; and then at 20-22 °C and 75 – 80 % humidity for 2-8 weeks. Cheese is then transferred to maturing store at 8-10 °C till marketed. In high alpine region (2500-3400 metres), it is stored under ambient condition. For first week, cheese is washed and turned every day, then twice a week for 3 weeks consecutively. The cheese develops good flavour after 5 months of ripening. Green cheese yield is 11% and 6-8 % of original weight is lost after 5 months curing (Thapa, 2000).

Chemical composition of 3 months old yak cheese reported as follows; Water 31.8%, TS 68.2%, butterfat on dry matter basis (BFDMD) 49.4%, salt 1.37% and pH 5.75; whereas three years old yak cheese; 23.1% water, 76.9% TS, BFDMB 46.8% and 3.12% salt. Similarly, two months old yak cheese produced by Nepalese private sector yak cheese producer contained: Moisture 39.0% by weight; TS 60.9% by weight; fat 27%; BFDMB 44.3% by weight; protein 27.4% by weight; lactose 1.9% by difference; and ash 4.5% by weight (Prajapati, 1996).

Kinema

Kinema is an indigenous, non-salted bacterial fermented soybean in the eastern hilly region of Nepal. It spreads to the north-eastern region of India including Bhutan and Hong Kong with the Nepalese community (G.C., 1994). The final product consists of sticky, colourless material accompanied by pungent odour of ammonia. The dominant organism responsible for the *Kinema* fermentation is *Bacillus subtilis*.

Soybean is cleaned, soaked, boiled, splitted, mixed with firewood ash, and packed with leaves (Banana etc.) in bamboo baskets and placed in warm place wrapped with clothes. Fermentation starts from the next day and goes for 2-3 days. The preparation of *Kinema* is very similar to Japanese *Natto*. An alternative method of improving the organoleptic and nutritional quality of *Kinema* could be the incorporation of maize (*Zea mays L.*) along with soybean; presence of maize could help in suppressing the ammonia odour as well as balanced amino acid profile specially cystine and methionine (Moktan, 2001). After fermentation, fresh *Kinema* is sun dried and stored for months. Ash is believed to increase the pH of boiled soybean to inhibit the growth of undesirable organisms like fungus while promoting the growth of *Bacillus* and increasing the mineral level in the final product

Sarkar, Tamang, Wok & Owens (1994) isolated 502 species of bacteria and yeast from market sample of *Kinema*. They have found that the sole fermenting organism for the production of *Kinema* is *Bacillus subtilis*. *Kinema* is fried with onion/garlic, chilli, and salt with other species and is served with beverages. It is also cooked by adding limited amount of other vegetables and meat making thick curry and is served with rice as an additional dish (Tamang & Sarker, 1988).Since *Kinema* serves as a major source of protein in the Nepalese diet, the distribution of amino acids in *Kinema* is an important nutritional issue.

Gundruk and Sinki

Gundruk is another popular fermented traditional food product used by the Nepalese and the Nepalese ethnic living elsewhere in the world since time immemorial. The fermenting substrate for *Gundruk* is *Rayo* (*Brasicca Campestris L*) leaves. Other leaves such as radish (*Raphanus Sativus L.*), *Shimerayo* (*Cardamine Hirsuta L.*), cauliflower (*Brasicca oleracea L.*) are also used as raw materials.

Gundruk is usually prepared during the months of November to February; a favourable climate when the weather is less humid and there is supply of raw materials. The preparation takes about 20-25 days. The leaves are selected, cleaned, washed, and withered in the sun for 1- 2 days depending on the weather. The leaves after mild crushing are placed in a bamboo pot or perforated tin and pressed by hands and then place heavy article (stones) to remove surplus water. It is placed on a dry sunny place for about 10-15 days. In a village where there is large amount of *Rayo*, radish, or mustard leaves available. *Gundruk* is prepared making a 1-meter hole dug on the ground with one-meter diameter and dried by burning fire to kill the microbes present and also to make warm. Then, the hole is covered by bamboo leaves followed by banana leaves (inside & outside) and placed the crushed leaves placing heavy stones for pressing. Left for 15-20 days for maturation-until fermented odour in developed. The *Gundruk* is taken out-sun dried for 2-3 days then packed. Shelf life of *Gundruk* is found to be about a year.
Gundruk is used as base for soup or as pickle. *Gundruk* soaked with water (for 2 minutes) and the water is squeezed and then fried with tomato, onion, chilli, salt etc. then added rice water or simply water served as hot soup as an appetiser. Gundruk pickle is made by soaking it in water and squeezing it and then adding salt, onion, chilli, and mustard oil, and is well mixed and is served. *Sinki* is similar to *Gundruk* except that the substrate is radish stem. Fermentation time may be 20-25 days little bit more than *Gundruk*. Consumption pattern is also similar to Gundruk

Achar

Achar (Pickles of various kinds) have been prepared in Nepal since time immemorial. Pickles of various kinds have been used in different country. Cabbage, Cauli flower, carrot, radish, chillies, ginger turmeric, rapeseed and its oil are the raw materials used. Mixed vegetable pickle is prepared by fermenting with dry salting method. The whole vegetables are withered in the sun for lowering the moisture content. Prepared vegetable pieces were packed in a glass bottle (half-kg). After bottling rapeseed oil was powered to cover the surface and capping the bottle left for fermentation 10 days.

Masyaura

Masyaura, a partially fermented legume-based savoury, is prepared from black gram dhal and colocasia. *Masyaura* also can be prepared using split black gram or green-gram with colocasia tuber ash gourd or radish depending upon their availability (Karki, 1986).Split blackgram dhal is washed thoroughly to remove the husk and foreign materials and soaked for 16 hour. It is then ground to make a thick paste. Colocasia tuber is also cleaned, washed, peeled, and finally shredded. Then shredded colocasia is well mixed with dhal paste in a ratio of 1:1. The dough is made into small lumps of 20-30 gram each distributed 1-2 inches apart on bamboo tray then sun dried 3 to 5 days,
Masyaura is a good source of protein (18-20%) carbohydrate (67-70%) and minerals. The protein quality of *Masyaura* is comparable to animal proteins Vitamins, especially thiamine, and riboflavin increase during sun drying. Calcium, phosphorus, and iron are the major minerals present in it (Dahal, Rao & Swamylingappa, 2003).*Saccharomyces cerevisiae* and *Candida versatitis* are the major organisms involved in fermentation, while principle molds involved are *Cladosporium* spp., *Penicillium* spp., and *Aspergillus niger* (Dahal, Rao & Swamylingappa, 2003). Dhal harbours the lactic acid bacteria in large numbers, which play a major role in fermentation contributing to special taste and texture.*Masyaura* are dried balls stored-for long time for future use. At the time of cooking, it is fried in oil, mixed with curry to make soup and is served with rice as a side dish.

Selroti

Selroti is a ring-shaped fried fare prepared by mixing rice flour paste [Rice: water 1:1 (w/v)], banana (1 small piece/ Kg paste), honey (5%), ghee (5%) and some spices. Sometimes banana and honey is replaced by sodium bicarbonate (0.25%) and sugar (10%). The well-mixed batter is allowed to ferment for 4 h (during summer) and 24 h (during winter). The kneaded batter is filled in a small funnel and deposited as continuous rings into hot oil. These rings are fried until brown and served while hot.

Mesu

Mesu is a pickle, which is prepared from the tender edible bamboo. The shoots are finely chopped (1×2 cm) and transferred into bamboo vessel or into a glass bottle, tightly packed and capped to provide an airtight environment. The material is allowed to ferment at ambient temperature (20-25°C) for 7 to 8 days. *Mesu* is usually prepared in the months of June to September. It has a sour taste and strong ammoniacal odour. A common pickle is also prepared by mixing *Mesu* with salt and green chillies. It is also used in the preparation of meat curry after frying.

Dahi and *Mahi*

Dahi is a Nepali word for Yoghurt, *Mahi* for buttermilk and *Ghiu* for ghee. *Dahi* is generally prepared from the boiled cow or buffalo milk by inoculating with a small quantity of starter *Dahi*. The cream is separated from *Dahi* by churning. The liquid portion is called *Mahi* (buttermilk) and is widely consumed as nutritious beverage.

Jandh and *Rakshi*

Jandh (beer type) and *Rakshi* (distillate) are major traditional alcoholic products of Nepal. *Jandh* is slightly acidic and sweet alcoholic beverage and *Rakshi* is the distillate from fermenting mash. These products are now prepared by small cottage industries. *Jandh* is a fermentation product of finger millet (*Eleusine coracana*) locally known as *Kodo* or *Marua*. The finger millet seeds are sometimes supplemented with a small amount of wheat or corn grains. The protocol for the preparation of *Jandh* and *Rakshi* is given below.

The millet seeds are steamed to make soft and spread on leaves, preferably of banana leaves. *Murcha*, the starter culture, is

powdered and sprinkled on the boiled and cooled seeds. After thorough mixing, the seeds are piled in a heap and kept for 24 h at ambient temperature. They are then usually placed in an earthen pot and covered with leaves and straw. In urban areas, the seeds are allowed to ferment in polyethylene bags. In case of millet, after fermentation, the seeds are kneaded to remove the seed coats. The grits are then placed in a bamboo vessels (*Toongba*) and water (cold or hot depending on the season). After 10 min, the beverage is ready to drink (normally with a bamboo straw). Generally, millet is mostly used to prepare *Jandh* while rice to prepare *Rakshi*. After fermentation, the mash is steam distilled in a traditional distillation apparatus.

Conclusions

Study on the production, processing, storage, and quality aspect of such an important indigenous fermented food products is limiting. Some of these popular and traditional fermented foods are gradually diminishing due to increased rate of urbanization, and indigenous technology not being transferred to the new generation or due to the lack of interest on the part of younger generation. It is a challenge to the food scientist and technologists not to miss out such an important history, as well as to help the ethnic community or region to improve the quality and quantity so that larger segment of consumers could be benefited.

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Post-Harvest Management for Export Marketing of Orange in Nepal

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This article presents a brief review on the post harvest management for export marketing of orange in Nepal. Proper harvesting, developed grading system, standard packing materials and methods, good handling practices, proper storage and display, good hygienic practices, sanitation and proper use of pesticides of citrus fruit has been suggested to minimize post harvest losses. Physicochemical characteristics of orange and their pricing system in Nepal are also reported in this article.

Keywords: Grading, Packaging, Storage, physicochemical characteristics, Pricing system

Introduction

Fruits and Vegetables, which are among the perishable commodities due to their physiology, are important ingredients in the human dietaries. Among fruits and vegetables, citrus fruit has vital role in human diet. Man has kept these commodities in his diet to provide the variety in taste and aesthetic appeal and to meet certain essential micro-nutrient like vitamin and ascorbic acid (vitamin C) The regular use of orange products keeps one healthy and strong and contributes towards longevity. Among citrus fruits, Mandarin orange has been identified as high value commodities by Agriculture Perspective Plan (APP) for production in mid hills of Nepal. This paper briefs the post-harvest management system for export marketing of orange in Nepal

Production of citrus fruits

World Citrus production is about 106,969 × 10^3 MT, whereas Nepal produces 1.31× 10^3 MT (Table 1). The production status of oranges in Nepal is shown in Table 2.

The post harvest physiology of fruits has in recent times become an important issue in both plant physiology and horticulture husbandry. The increase attention accorded to post harvest technology of these commodities is mainly due to the realization that faulty handling practices after harvest can cause to large input of labor, input and capital to produce. It is reported that post harvest losses in fruits, especially in orange, are about 20-30% in our country affecting the lowering of the market value and consumer's acceptability. Improper handling and storage cause physical damage due to tissue breakdown.

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Table 1: Citrus Fruit Production in Nepal

Countries	Total Production (×10^3 MT)	Contribution % with	
		(Asia-Pacific)	World
Bangladesh	37	0.1	0.03
Bhutan	58	0.2	0.05
India	4,258	15.2	3.98
Nepal	131	0.4	0.12
Pakistan	1,870	6.7	1.75
Sri Lanka	23	0.1	0.02
World	106,969		100

(Source: FAO/UN-2001, Data represent the production at 2000)

Table 2: Production of orange in Nepal (2001)

Development Region	Production Area (Hectare)	Productivity (MT/Hectare)
Eastern	1,915.7	11.4
Central	1,364.8	11.1
Western	2,602.8	11.1
Mid Western	993.1	9.1
Far Western	491.8	10.2
Total	7,368.5	

(Source: Annual Report Bulletin-2001)

Maturity-A measure of quality for citrus fruit

The post-harvest quality and storage life of fruits are controlled by maturity. If fruits are harvest at proper stage of maturity; their quality will be excellent. External color of the fruit, its juiciness and flavor, total soluble solid (TSS) and Brix /Acid ratio (B/A) are the main criteria for judging the maturity of fruits. However, no maturity standards for picking citrus fruit in Nepal are developed, whereas in many developed countries regulation has existed for controlling the stage at which fruits should be harvested. The neighboring country (India) has

established the voluntary standard as reference to determine the stage of maturity as follow (Table 3).

Table 3: Maturity Standards for orange

Parameter	Standard Range
Juice (Not less than)	42 %
Acid	0.4-0.7 % as citric
Brix/Acid ratio	16.0 and below (Poor quality)
	16.1 – 20.0 (Good quality)
	20.1 – 24.0 (Special quality)
	24.1 and above (Sweet only)

Post Harvest Management of Orange in Nepal

Team of ABTRACO has reviewed and studied the post harvest management trends and factor influencing the quality of orange so that the recommendation can be made for export market. Some changes after harvest is essential for the attainment of the desired degree of eating quality. Trends in the marketing of orange fruits are that the fruits are picked at mature-green stage of development and then allowed to ripen off the plant to attain optimum eating quality. The recommended post harvest handling system of oranges for export market has been illustrated in Figure 1 *Harvesting*

Mechanical damage during harvesting and subsequent handling operation can result defects in the produce and permit invasion by disease causing microorganism. The inclusion of dirt from the field can aggravate this situation. Produce can overheat and rapidly deteriorate during the temporary field storage. Failure to short and discard immature, overripe, under sized, misshapen, blemished or otherwise damaged produce creates problems in the subsequent marketing of the produce.

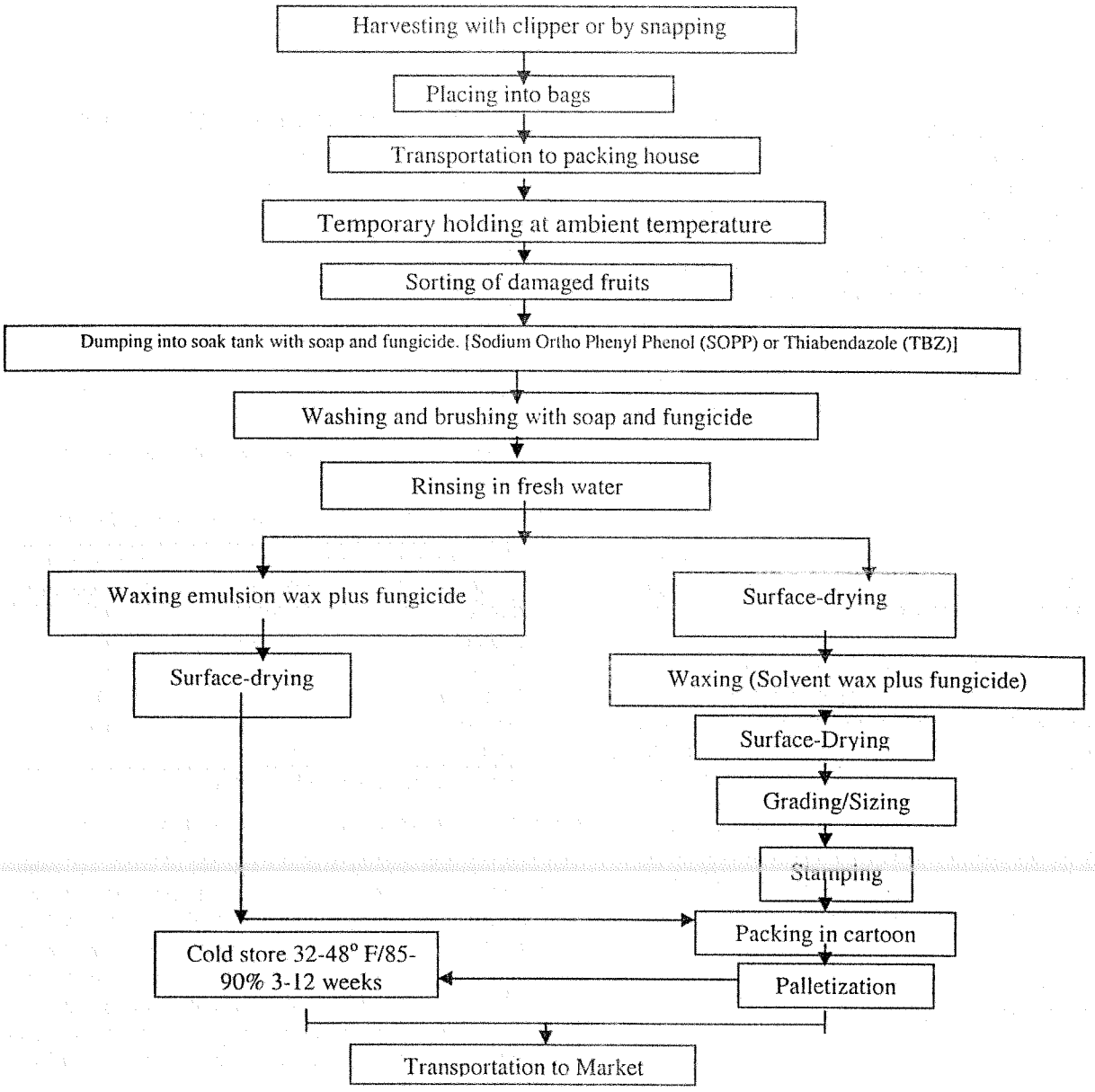

Figure 1: Post Harvest handling systems for oranges

During the period of season, the project team has studied about the traditional methods of harvesting and harvesting time and maturity stage. It was found that most of the farmers used to harvest orange at the time when the traders come and in their presence. The actual harvesting time, methods and maturity stage have been identified to have the better quality. This package of technology will be suggested the coming up seasons and disseminated in the study area.

The fruits are harvested at mature stage and allowed to ripen during transit, storage or displayed in retail shops. When the green color changes to yellow about or more than 50%, this is the right time for harvesting. In some of the areas, it is also found that orange is harvested when the orange becomes complete deep yellow. Generally, oranges are harvested by hand picking. There are losses of 2-5% during harvesting.

Sorting/Grading/Packaging

Sorting and grading plays key role for selecting the quality and uniform size of fruits and vegetables. There is no sorting and grading system in farmer's level, but some of the traders are using grading size. There is no grade standard in Nepalese context. Here, we have observed different size of oranges of different places and categorized as extra-large, large, medium and small according to their sizes (Table 7)

Improper packaging system and methods creates the quality problem and affects the quality and post harvest losses. The choice of package and packing methods must take account. The dimension of the package i.e. the size and shape should provide economic use of materials, adequate strength and easy and secure handling, loading and stacking. The optimal length/width ratio of about 1.5:1 made of hard board corrugator's carton (14 cm x 25 cm x 20 cm) with holes of 1-cm diameter and cutting paper as cushion are suggested for packaging the oranges. Alternate bamboo stack package is also suitable for transporting the orange for better quality and less damage and injuring. However, it is learnt that Bangladesh wholesalers prepare wooden boxes with ventilation spaces

Bamboo basket (*Doko*) of 30-50 kg capacity is normally used for packing and carrying the oranges. Harvested oranges are packed in bamboo baskets using straw as a cushioning material, which protects oranges from damage during transportation. Farmers and traders don't use carton boxes, wooden boxes for packing oranges, but few traders used plastic crates (20-25 kg.) and polythene bags (20-22 kg.) for packing oranges.

Transport and Handling

Generally in the orange growing area harvested oranges are transported from orchard to road (collection center) by porter using bamboo basket (*Doko*). The traders collect the orange from farmers and transported by truck or buses using plastic crates or polythene bags to the market areas. It was also known that the oranges were loaded in trucks without packing but keeping straws in the bottom layer and transported. The losses during transportation from orchard to road vary from 4 to 8 % and losses from collection center to market vary from 5 to 10 %. During transportation, damage occurring due to rough handling, vibration, bursting etc. can be minimized by using high stacking strength of package, using cushion materials, proper handling during loading and unloading.

Storage

Storage of fresh produce in Nepal seems to have evolved out of a necessity to cushion the effect of irregular delivery rates to wholesaler. Consequently, the ambient Air Storage facilities, which could be secured from pilferages, were logically the first steps towards development of storage facilities. Although Nepalese prefer fresh produce in season, some of the private entrepreneurs have sought profit from the demand of export and urban market and have built up cold storage facilities in or near major market centers. The total capacity of 14,500 tons of storage facilities existed in the country and was expected to increase. In addition to cold storage there are limited facilities for cellar storage (no cooling equipment) and cool chambers (using evaporations cooling). These facilities are found in most of the region of Nepal to store fruits and vegetable.

Harvested oranges are immediately sent to the markets, as there is no storage system in the farmer's level or in orange growing areas. There is few cellar store capacity (2-3 MT) in some of the districts, but they are not properly utilized. In some of the orange growing areas, late harvesting is one of the main reasons for storing oranges in the tree itself.

Post Harvest losses of orange in Nepal

Post harvest losses in quality are substantial and depend up the products where it is produced and where it is finally consumed. The losses are varying from 10 to 30 percent from one place to another. Post harvest losses from Dhankuta (A city in the eastern Nepal) to Kathmandu (The capital city) by bus and porter have been studied and assessment for the loss from farm to the market area made. The loss during study period is summarized in Table 4.

Table 4: Post Harvest losses of orange at different holding points in Nepal

Particulars	Holding period (Days)	Quantity (Kg.)	Loss (%)
Farm	1-2	150	-
Farm to collection center (Dhankuta)	1-2	142.5	5.0
	3-4	134.2	10.5
Collection center to wholesaler (Kathmandu)	4-5	127.8	14.8
Wholesaler to retailer	1-2	120.5	19.6
Retailer to consumer			

(Source: ABTRACO, 2000)

Grading and pricing system of orange in Nepal

Grading

There is no practical system of orange grading in the country. A report published by Horticulture Development Project, Kirtipur, Bulletin No. 11, 1997 has recommended the following grading size.

Table 5: Recommended grading size

Size	Diameter (mm)	Avg Wt/Fruit (gm)
LL Size	76 and above	140
Large Size	70 – 75	115
Medium	65 – 69	100
Small Size	59 – 64	78
SS Size	Below 58	60

(Source: HDP, 1997)

Orange sample collected from different orange areas in Nepal like Dhanakuta, Dhading, Gorkha, Lamjung and Tanahun to determine the size of orange (diameter) number of oranges per Kg, Juice content, total soluble solid (TSS), total acidity, skin % and seed %. The results are given in Table 6.

The size of oranges for extra large varies from 71–75 mm, large size varies from 66–70 mm, medium size varies from 51–58 mm and small size varies from 45–50. The number of orange per kg for extra large varies 6–8, for large varies 7–9, for medium 10–12 and for small 14–18. Percent of skin varies from 22.2-37.5%, seed varies from 1.7- 2.5% juice varies from 35.5-50.1%, total soluble solids varies from 10.0-13.8 %, total acidity as citric acid varies from 0.41-0.75 %. The orange from Tanahu (Bandipur) has less juice content and high skin as compared to other. These sizes of oranges were taken within the available of orange sample. So this sizes are little bit differ from the recommended size of orange. It may be due to smaller size of sample during study. The categorized oranges as extra large, large and medium are better for export quality. The small oranges may either be locally consumed or used for processing. Orange grown in eastern part of Nepal is superior to those of the western part of Nepal in the context of TSS and acidity. The oranges collected from India showed the percent of skin, juice, TSS and total acidity are similar to Nepalese orange, except percent of seed is less in Nagpur orange.

Table 6: Physico-chemical characteristic of oranges in Nepal

Date	Place	Diameter (mm)	Orange Kg	Skin %	Seed %	Juic %	TSS %	Acidity % (Citric)
2001/12/01	Gorkha (Manakamana)							
	Extra-Large	71-75	6-7	26.2	2.1	48.3	10.0	0.70
	Large	66-70	8-9	28.0	1.8	47.8	10.2	0.71
"	Gorkha (Bunkot)			27.3	1.7	45.4	10.0	0.59
	Large	66-70	7-8	26.6	2.2	45.0	11.2	0.89
	Medium	56-65	9-10					
"	Lamjung (Udaipur)							
	Extra-Large	70-73	6-7	25.5	2.3	47.0	10.4	0.56
	Large	66-70	8-9	26.0	2.1	48.8	10.5	0.56
2002/01/14	Dhankuta (Khoku)							
	Extra-Large	71-73	6-7	27.8	2.2	46.2	11.4	0.55
	Large	65-67	8-9	23.2	2.0	50.1	12.4	0.67
	Medium	51-56	11-12	22.1	2.5	49.8	11.6	0.58
	Small	45-55	16-17	22.2	1.7	48.5	13.2	0.56
2002/01/16	Tanahun (Bandipur)							
	Extra-Large	71-73	7-8	31.9	1.9	40.8	11.0	0.41
	Large	65-68	9-10	34.2	1.7	35.5	13.2	0.49
	Medium	53-58	12-13	26.4	2.5	37.4	12.4	0.57
	Small	47-51	17-18	37.5	1.9	36.1	12.0	0.70
	Dhading (Syardul)							
	Large	66-70	8-9	28.8	2.5	41.4	11.4	0.50
2002/01/23	Medium	58-62	9-10	25.2	1.8	45.7	12.4	0.55
	Small	52-56	14-15	28.6	1.9	35.0	13.8	0.75
	Nagpur (India)							
	Extra-Large	72-77	6-7	29.8	0.8	45.8	12.0	0.54
2002/3/12	Large	67-70	7-8	25.2	1.4	41.9	12.2	0.65
	Medium	62-65	8-9	24.3	0.6	44.4	10.8	0.69
	Small	52-59	12-13	29.3	1.1	36.3	11.2	0.74

(Source: ABTRACO, 2000)

Table 7: Comparative characteristics of Nepalese orange with Indian (Nagpur) orange

Particular	Chemical Characteristics		Standard
	Nepalese	Indian	(ISI)
Juice %	43.7	42.1	42.0
Acidity % (Citric)	0.59	0.66	0.40–0.70
T.S.S (° Brix)	11.6	11.5	6.4– 14.4
Brix/Acid Ratio	19.7	17.5	16.1– 20.2

Pricing System

Generally, market price of orange is determined by the force of demand and supply. The system of fixation of price is done by negotiation. Wholesalers fix the price of the products of the whole orchard on contact basis prior to the harvesting period. Those wholesalers generally collect big and small quantities of oranges from different contracting farmers and distribute to retailers at agreed prices. Farmers are generally weak in bargaining and economic compulsion forces them to accept the prices offered by the wholesalers. Table 8 indicates prices and margin recently being made at various points along the marketing period.

Table 8: Price structure of oranges by market components

Component	Prices (NRs/100Kg)	Margin (%) on consumer's price	Mark up on purchase price
Farmer receives	1,300	43.6	0.0
Assembler/Broker receives	1,404	3.5	8.0
Wholesaler's cost			
Transport	24		
Handling/packaging	120		
Taxes	150		
Weight loss in Transit (5%)	65		
Damage/Wastage (5%)	65		
Storage (1-2 Weeks)	50		
Overhead Cost (7.5%)	98		
Profit (5%)	65		
Wholesaler receives	2,041	21.4	49.0
Retailer's Cost			
Storage (2-3 weeks)	130		
Shop rent	75		
Handling loss (25%)	325		
Labour	150		
Overhead Cost (10%)	130		
Profit (10%)	130		
Retailer receives	2,981	31.5	72.3

(Source: ABTRACO, 2000)

These figures confirm that farmers of course receive the largest share of the final selling prices but this is only 43%. The figure itself indicates the maximum share has gone to the wholesalers and then to the retailers that consumers have to pay.

Conclusions

As fruits and vegetables are considered as important high value commodities, there are immense scopes for cultivation in potential hill area of Nepal. There are considerable demands of fresh fruits especially orange in local market as well as Tibet and Bangladesh. However, due to their short shelf life and poor keeping quality, its marketability is constrained. With the aid of present knowledge in the areas of pre and post harvest management, shelf-life and quality of the produce can be improved. Improved system of packaging, handling, storage conditions and transportation as suggested above can extents of the shelf-life and improve the keeping quality, which aids to long distant transportation for export purpose.

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Safety Aspects of Biogenic Amines in Cheese

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Biogenic amines are natural antinutritional factors and are important from a hygienic point of view as they have been implicated as the causative agents in a number of food poisoning episodes, and they are able to initiate various pharmacological reactions. Among the amines, tyramine, histamine, β-phenylethylamine, putrescine, cadaverine, and tryptamine are the most often occurring biogenic amines in cheeses. Histamine is considered as a causative agent for food poisoning outbreaks while tyramine and the toxicity of histamine is enhanced by the presence of other amines such as cadaverine and putrescine. Tyramine is associated with the hypertensive crisis also known as "cheese reaction". Biogenic amines may also be considered as carcinogens because of their ability to react with nitrites to form potentially carcinogenic nitrosamines. The toxic threshold of biogenic amines depends on the individual's physiological condition and other factors.

Keywords: Biogenic Amine, Biosyathesis, Safety, Cheese

Introduction

Biogenic amines are low molecular weight organic basic nitrogenous compounds, in which one, two or three atoms of hydrogen in ammonia are replaced by alkyl or aryl groups, and possessing biological activity. As these amines are formed by the action of living organisms through enzymatic decarboxylation of free amino acids, they are designated biogenic (Halasz et. al, 1994; Shalaby, 1996; Hernandez-Jover et al, 1996; Vale & Gloria, 1998). Biogenic amines are classified as aromatic biogenic amines (tyramine, histamine, tryptamine, β-phenylethylamine, octopamine, dopamine, and serotonin), diamines (putrescine and cadaverine), and polyamines (agmatine, spermidine, and spermine) (Joosten & Olieman 1986), among them, tyramine, histamine, putrescine, cadaverine, tryptamine, and β-phenylethylamine are the most important biogenic amines occurring in cheeses (Joosten, 1988; Tawfik et al., 1992; Santos, 1996). They are toxic substances which cause diseases in man and animal, where toxaemias resulting from ingestion of food containing biogenic amines have been reported in the world (Table 1). Foods that have been prepared by a fermentative process, or have been exposed to microbial contamination during aging or storage, are likely to contain biogenic amines. Foods possibly to contain biogenic amines include fish, fish products, meat products, eggs, cheeses, fermented vegetables and soy bean products, beers and wines (Shalaby, 1996). After fish, cheese is the next most commonly implicated food associated with histamine poisoning (Stratton et. al, 1991). Many scientists found proved that cheeses represent an ideal environment for the formation of free amino acids and biogenic amines (Vale & Gloria, 1997; Vale and Gloria, 1998; Edwards & Sandine, 1981; Chang et al., 1985)

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Table 1: Amine poisoning occurring in certain countries in the world

Country	*Food incriminated*
Canada	Fish, Cheddar cheese
Denmark	Fish
France	Fish, Swiss cheese, Cheshire, Gruyere
Germany	Fish, Gouda cheese, Sauerkraut
Great Britain	Fish
Japan	Fish, Chicken
Netherlands	Gouda cheese
New Zealand	Fish

(Source: Joosten, 1988; Tawfik et al., 1992; Santos, 1996)

Biosynthesis of Biogenic Amines

Biogenic amines are mainly generated by the enzymatic decarboxylation of amino acids by micro-organisms (Valsamaki et al., 2000 & Shalaby, 1996) during aging and storage of cheese (Shalaby, 1996). During cheese ripening, casein is slowly degraded by proteolytic enzymes, leading to an increase of peptides and free amino acids content (Foster et al., 1958; Joosten & Olieman, 1986), which both serves as precursors for amine formation by specific bacterial decarboxylases, in presence of amino acid decarboxylase-positive microorganisms.and formed amines and carbondioxide (Degheidi et al., 1992; Leuschner, et al. 1999). Amine build-up usually results from decarboxylation of free amino acid by enzymes of bacterial origin. Amino acid decarboxylation takes place by removal of the α-carboxyl group to give the corresponding amine. Arginine is decarboxylated to produce agmatine; lysine is decarboxylated to produce cadeverine. Similarly, histidine, tyrosine, tryptophan, and phenylalanine are decarboxylated to histamine, tyramine, tryptamine, and β-phenylethylamine (Shalaby, 1996). Two mechanisms of action, for amino acid decarboxylation, have been identified; a pyridoxal phosphate dependent reaction and a non-pyridoxal

phosphate dependent reaction. In cheese, polyamines remained relatively constant throughout ripening because of the fact that they are natural amines and, therefore, not produced by microbial decarboxylation activity (Bardocz 1993, 1995).

Factors Affecting the Formation of Biogenic Amines in Cheese

Cheese represents an ideal environment for amine production but the formation of amine depends on different factors. Prerequisites for biogenic amine formation by microorganisms are:

- ✓ Availability of free amino acids, but not always leading to amine production (Marklinder & Lonner, 1992).
- ✓ Presence of decarboxylasa-positive microorganisms (Brink et al., 1990).
- ✓ Conditions that allow bacterial growth, decarboxylase synthesis and decarboxylase activity (Brink et al., 1990).

The precursors of the main biogenic amines involved in food poisoning are: (Santos, 1996)

Histidine – histamine	Tyrosine – tyramine
Hydroxytryptophane – serotonin	Tyrptophane – tryptamine
Lysine – cadaverine	Ornithine – putrescine
Arginine – spermine	Arginine – spermidine

Presence of Free Amino Acids

One of the most important factors influencing the formation of biogenic amines in cheese is the presence of precursor free amino acids (Eitenmiller et al., 1978). These amine precursors arise via proteolysis. Sumner et al., (1990) found that histamine formation seems to be dependent of the degree of proteolysis. The accumulation of free amino acids can be mainly attributed to the hydrolytic activities of several enzymes, such as proteinases from milk, milk-clotting enzymes and proteolytic enzymes from starter and non-starter microorganisms. In addition, several factors such as high temperature, high pH and low salt content have been reported to accelerate the amino acid accumulation and, hence, stimulate amine formation (Joosten, 1988). During cheese ripening processes the activity of the decarboxylating enzymes could be a more important limiting factor than the precursor availability (Edward & Sandine, 1981; Joosten & Boeckel, 1988). Cheeses were observed to contain ample pyridoxal phosphate which is required for amino acid decarboxylase activity (Shanani et al., 1962).

Bacterial Strains

Numerous bacteria, both intentional and adventitious, have been reported as being capable of amine production which includes *Escherichia, Enterobacer, Salmonella, Shigella, Clostridium perfringens, Streptococcus faecium, Streptococcus mitis, Lactobacillus bulgaricus, Lactobacillus plantarum, Lactobacillus casei, Llactobacillus acidophilus, Lactobacillus arabinose* and *Leuconostoc* (Edwards & Sandine, 1981; Chang et al., 1985; Stratton et al., 1991). *Enterococci* are considered to be notorious tyramine formers. Representative of the *Enterobacteriaceae* can cause cadaverine and putrescine build-up even at low densities (Joosten & Northolt, 1987). Therefore, biogenic amines might be useful indicators of spoilage (Halasz et al., 1994). Some starter cultures bacteria in the dairy industry, such as *Streptococcus lactis* and *Lactobacillus helviticus*, are identified as histamine producers (Stratton et al., 1991). But most often, the non starter lactic acid bacteria and *Enterobacteriaceae* are taken responsible for the production of biogenic amines in cheese (Joosten & Northolt, 1987). Kebary et al., (1999) reported that addition of lactobacilli prior to cheese-making (to accelerate cheese ripening) increases the formation of biogenic amines and free amino acids; *Lactobacillus helveticus* was found to be more effective for increasing the formation of biogenic amines and liberation of free amino acids than *Lactobacillus casei*. Leuschner, Kurihara & Hammes (1999) reported that the presence of two strains of *Enterococcus faecalis* ssp *liquefaciens* during cheese fermentation resulted in high tyramine concentrations (477 $mgkg^{-1}$) in the end product after a 12 week ripening period. Non-starter bacteria present in cheese are important for biogenic amine formation in cheese (Novella-Rodriguez, et al., 2002).

pH

pH of cheese also has a pronounced effect on the formation of biogenic amines. According to Edwards & Sandine (1981), the pH of cheese is appropriate for amine production, generally between 5.0 and 6.5, depending on age and type. Amino acid decarboxylase activities were higher when pH ranged between 4 and 5.5. However, amine formation was found to depend on the growth activity of bacteria rather than on the growth conditions per se (Yoshinaga & Frank, 1982) Koessler et al. in 1928 initially suggested that amine formation by bacteria was a physiological mechanism to counteract acid environment which was found to be proved by Gale, (1946) and Chander, et al., (1988). Gardini et al., (2001) reported that the biogenic amine production was every low at the higher NaCl concentrations and this trend was enhance by the lower pH; the increase of pH resulted in a rapid increase in biogenic amine production. Maijala et al. (1993) and Maijala (1994) also reported that biogenic amine production by *Enterococcus faecalis* EF37 is significantly decreased with decreasing pH. Vale and Gloria (1998) reported that among other quality parameters, acidity influenced amine formation in several types of cheeses and these results were supported by the theory that the formation of biogenic amines is a protective mechanism of bacteria against acidic environments (Eitenmiller et al., 1981; Maijala, 1994).

Temperature

With respect to the influence of temperature on the synthesis of biogenic amines, some authors found that storage temperature did not significantly influence maximum content of certain biogenic amines, though refrigeration temperatures delayed the onset of such increase (Santos, 1996; Gardini, et al., 2001). However, the role of temperature should not be underestimated.

It is well known that temperature has a marked effect on the formation of biogenic amines in cheese. Several researchers reported that amine content depends on temperature, and increases with time and storage temperature (Halasz et al., 1994). In fact, temperature had different influences on phenomena endowed with the biogenic amine production, such as the growth kinetics and the cell yield. Moreoever, its effects on the activity of proteolytic and decarboxylase enzymes and the relationships between the microbial populations (Joosten & Van, 1988; Maijala et al., 1995) play an important role in relation to the total amount of amine produced. Therefore, biogenic amines are gradually increased by extending cheese ripening time (Degheidi et al., 1992).

Pinho, et al. (2001) reported that the content of free amino acids and biogenic amines remained almost the same for the Azeitao cheese stored at 4^0C while at the room temperature, the content of both free amino acid and biogenic amine increased significantly. Moreover, El-Sayed, (1996) reported that processed cheeses which are subjected to high temperature during manufacturing contained appreciable levels of biogenic amines.

Other Factors

The heat treatment of the milk along with the fat content also affects the formation of biogenic amines in cheese. Total biogenic amines of cheeses made from 3% milk fat increased by heating the milk up to 70^0C, while they decreased by raising the heat treatments to 75^0C and 80^0C. But, for milk with 2% fat, the biogenic amine increased up to 75^0C treatment. Also, the concentration of total biogenic amines decreased as the fat content of cheese milk was decreased, which might be due to the decrease of water activity (Kebary, et. al., 1999).

The lower the NaCl concentration, the higher will be the amine production. A 5% NaCl concentration minimizes the presence of amine, but this value can be lowered with a decrease in pH (Sumner et al., 1990; Joosten, 1988 and Gardini et al., 2001). Chander et al. (1989) found that the rate of amine production by a *Lactobacillus bulgaricus* strain was considerably reduced when salt concentration in the medium increased from 0 to 6%. This negative influence can be attributed either to reduced cell yield obtained in the presence of high NaCl concentrations and / or to a progressive disturbance of the membrane located microbial decarboxylase enzymes (Sumner et al., 1990). Other factors such as poor hygienic quality and the length of ripening also contribute in biogenic amine formation (Buffa et al., 2001).

Physiological Effects of Biogenic Amines in Human

Amines such as histamine accumulate in cheeses during aging (Antila et al., 1984; Stratton et al., 1991). Histamine, tryptamine, β-phenylehtylamine, and tyramine are biologically active amines found in cheese and which have important physiological effects in humans, and generally are either psychoactive or vasoactive. Psychoactive amines affect the nervous system by acting on neural transmitters, while vasoactive amines act on the vascular system. Amines such as polyamines, putrescine, spermidine, spermine and also cadaverine are indispensable components of living cells and are important in the regulation of nucleic acid function and protein synthesis and probably also in the stabilization of membranes (Bardocz et al., 1993; Maijala et al., 1993; Halasz et al., 1994)

The consumption of food containing biogenic amines is also responsible for many pharmacological effects (Table 2) which lead to several types of food borne disease, including histamine poisoning (scombroid poisoning) and tyramine toxicity (cheese reaction). Harmful effects resulting from the consumption of foods rich in biogenic amines can be expected only when these amines gain access to the bloodstream (Joosten, 1988). Moreover, these compounds can represent a serious health hazard for humans and animals when present in food in significant amounts, or ingested in the presence of potentiating factors, such as monoamine oxidase-inhibiting (MAOI) drugs, alcohol, and gastrointestinal diseases (Stratton et al., 1991). Furthermore, in the presence of nitrite, these amines may form N-nitosamines, some of which are known to be carcinogenic, mutagenic, teratogenic and embriophatic (Oliveira et al., 1995).

Histamine, putrescine and cadaverine are often documented in clinical studies with histamine being linked to food poisoning and putrescine and cadaverine potentiating the toxicity of histamine (Eitenmiller et al. 1980). Histamine has an important role in human metabolism, such as the release of stomach acid. The presence of low levels of biogenic amines in cheeses is not considered a serious risk. However, if normal routes of amine catabolism are inhibited or the amount consumed is large, various physiological effects may result (Koehler & Eitenmiller, 1978; Taylor, 1986).

The intestinal tract of humans contains the enzymes diamine oxidase (DAO) and histamine-N-methyl transferase (HMT) which convert histamine to harmless degradation products. Putrescine and cadaverine can inhibit these enzymic reactions and are therefore potentiators of histamine toxicity. Other biogenic amines that may act as potentiators of histamine toxicity include tyramine, tryptamine, and β-phenylethylamine (Stratton et al., 1991). Spermine & Spermidine also increase the histamine transport across the gastrointestinal wall (Jung & Bjeldanes, 1979; Chu & Bjeldanes, 1981). The presence of low levels of histamine in the diet normally has no toxic effect as humans do not absorb histamine efficiently from the gastrointestinal tract. If a high level of histamine is present in the diet, then the capacity of DAO and HMT to detoxify histamine will be limited and histamine will enter into the bloodstream resulting in histamine poisoning. Histamine acts by dilating blood cells and can result in hypotension (Taylor, 1986).

Table 2: Biogenic amines in foods and their pharmacological effects

Amine	*Precursor*	*Pharmacological effects*
Histamine	Histidine	-Liberates adrenaline and noradrenalin -Excites the smooth muscles of the uterus, the intestine, and the respiratory tract -Stimulates both sensory and motor neurons -Controls gastric acid secretion
Tyramine	Tyrosine	-Peripheral vasoconstriction -Increases the cardiac output -Causes lacrimation and salvation -Increases blood sugar level -Releases noradrenalin from the sympathetic nervous system -Causes migraine
Putrescine and cadaverine	Ornithine and lysine	-Hypotension -Bradycardia -Lockjaw -Paresis of the extremities -Potentiate the toxicity of other amines
β-phenyethylamine	Phenylalanine	-Releases noradrenaline from the sympathetic nervous system -Increases the blood pressure -Causes migraine
Tryptamine	Tryptophane	-Increases the blood pressure

(Source: Shalaby, 1996)

The incubation period of histamine poisoning is short. Following ingestion of a meal containing high levels of histamine, poisoning effects can occur within several minutes to a few hours (Taylor, 1986). The duration of illness is usually short (Taylor & Bush, 1988). Histamine poisoning is often manifested by a wide variety of symptoms. Characteristic symptoms affecting the cutaneous (i.e. skin) system include rash, urticaria, edema, and localized inflammation (Stratton et al., 1991; Taylor, 1986). Gastrointestinal involvement is characterized by nausea, vomiting, diarrhea, and abdominal crampt (Taylor, 1986). Other symptoms include hypotension, headache, palpitation, tingling and flushing (Taylor, 1985). In severe and burning sensation cases, bronchospasms, suffocation and severe respiratory distress have been reported (Franzen & Eysell, 1969). Cheeses associated with histamine poisoning are Gauda, Swiss, Cheddar, Gruyere, and Cheshire (Sumner et al., 1985; Taylor, 1985).

Tyramine, tryptamine, and β-phenylethylamine are included in the presser amine group which are vasoactive amines. The importance of tyramine in foods is principally due to its toxicological implications since apart from being slightly toxic in itself; the tyramine reacts with monoamine oxidase inhibitor (MAOI) drugs giving rise to hypertensive crisis. Cheese was the food initially associated with hypertensive disturbances noted in patients undergoing treatment with MAOI. The increase in blood pressure, observed after ingestion of foods rich in tyramine, is known as the "cheese reaction" which can cause severe headache and may induce a brain haemorrhage or heart failure (Smith, 1980; Koehler & Eitenmiller, 1978; Edward & Sandine, 1981).

The exact toxic threshold of biogenic amines is difficult to determine due to its dependence on the efficiency of detoxification mechanisms of different individuals. However, toxic level of different biogenic amines has been investigated by different researchers. According to Chang et al. (1985) and Halasz et al. (1994), 10 mg of histamine in 100 gm of sample can cause histamine poisoning; 10 – 80 mg of tyramine can cause "cheese reaction" (6 mg if patient is receiving MAOI); and 3 mg of β-phenylethylamine can cause migraine headache. Histamine at a concentration of 8–40 mg can cause slight, over 40 mg, moderate, and over 100 mg, severe poisoning whereas 10 - 80 mg tyramine can cause toxic swelling and over 100 mg may cause migraine. Also, in fish, <5mg per 100 g is safe for consumption, 5 – 20 mg per 100 g, probably toxic and >100 mg per 100 g, toxic and unsafe for consumption. Ayres et al. (1980) reported that an intake of >40 mg biogenic amines per meal has been potentially toxic. From a "Good Manufacturing Practice" point of view, levels of 50 – 100 ppm histamine, 100 – 800 ppm tyramine and 30 ppm β-phenylethylamine or a total of 100 – 200 ppm are regarded as acceptable (Nout, 1994). But the toxic threshold level of tryptamine is not known (Joosten, 1988).

Control of Biogenic Amines Formation in Cheese

Vale & Gloria (1998) reported that the amines were not detected in every sample and the high variability on amine levels among samples of the same type of cheese suggest that the presence of amine in cheeses can be prevented. Therefore, the sources and critical control points for amine formation during cheese manufacture should be determined in order to limit amine formation and accumulation in cheese. Use of good hygienic conditions during the manufacture together with good quality raw material with low total counts of contaminant microorganisms and free of amine-producing microorganisms is the preventive measures to control the formation of biogenic amines in cheese (Galgano et al., 2001). Many authors (Chang et al., 1985; Joosten, 1988; Giraffa et al., 1995) reported that the production of biogenic amines in cheese could be limited by the amount of their precursor, i. e., the free amino acids in the medium. The best cheese-making condition reported by Gennaro, et al., (2003) was pasteurized milk, mesophilic starters and heated curd.

Food additives such as potassium sorbate may be used to limit the formation of biogenic amines in foods (Shalaby, 1996). Moreover, susceptible individuals should be advised to consume cheeses with low biogenic amines contents (Vale & Gloria, 1998). Galgano et al. (2001) reported that strains with low or no amino decarboxylase activity could be selected as starters both to control the tyramine, 2-phenylethylamine and tryptamine contents of Semicotto Caprino cheese. However, the use of selective starter cultures did not reduce the formation of histamine and tyramine, even when the amine-producing microorganisms were present in very low concentrations. Joosten and Nunez (1996) reported that the use of bacteriocin-producing starters prevented the formation of histamine in cheese. High pressure treatment (400 MPa) of cheeses for acceleration of ripening did not have effect nor even reduce the total biogenic amine content of cheeses; so, the high pressure treatment could be helpful to obtain cheeses with low biogenic amine contents (Novella-Rodriguez & Veciana-Nogues, 2002). Novella-Rodriguez et al., (2002) reported that the high pressure treatment of milk for cheese making did not yield higher production of undesirable biogenic amines.

Conclusions

Cheese made with raw milk is highly perceptible and has the required flavour profile than that made from treated (pasteurized or ultra-filtered). But, these non-starter microorganisms may be responsible for the formation of biogenic amines in cheese depending upon the decarboxylase activity. So, heavy contamination of cheese by non starter microorganism should be avoided by strict use of good hygiene in both raw material and manufacturing environment.

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Physicochemical and Biochemical Changes during Ripening of Dry Fermented Chinese-Style Sausages Inoculated with Lactic Acid Bacteria

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Dry fermented Chinese-style sausages were prepared by inoculating two strains of lactic acid bacteria (LAB): Lactobacillus casei subsp.casei-1.001 and Pediococcus pentosaceus-ATCC 33316. Changes in physico-chemical and biochemical characteristics were determined during ripening of sausages. A significant ($p<0.05$) drop in moisture content of sausages inoculated with LAB caused to reduce water activity (below 0.83). Likewise, a significant ($p<0.05$) decline in pH (4.23) and nitrite level (2.0 mg/kg) was observed in ripened sausage inoculated with L. casei subsp.casei-1.001. Furthermore, red color value (a =10.68 unit) as well hardness and chewiness were also found to be significantly ($p<0.05$) increased in that sample. In contrast, TBA (1.05mgMDA/kg) value and hardness value were significantly ($p<0.05$) become lower in product inoculated with P. pentosaceus-ATCC 33316. Both of LAB starters showed insignificant effects ($p>0.05$) on TVBN and NPN values. As compare to sausage sample inoculated with L. casei subsp.casei 1.001, a higher concentrations of free amino acids (1228.19 mg/100g dm) as well as total biogenic amine (405.8mg/kg) were detected in finished product inoculated with P. pentosaceus-ATCC 33316; however, the amount of biogenic amines were lower than the values obtained for control.

Keywords: Dry fermented Chinese-style sausage, Lactic acid bacteria (LAB), Ripening, Free amino acid, Biogenic amine

Introduction

Chinese-style sausage is one of the most popular traditional meat products in China. Traditionally, it is prepared by blending ground pork meat and fat with wine, sugar, salt, soyasauce and other spices and then filled into pork small intestine, which finally dried in air or sun especially in winter season. Chinese-style sausages are 'semi-dry' or 'short-time fermented sausage' and differ from western-style dry fermented sausages in manufacturing process, ingredients, bacterial ecology and flavor (Guo, & Chen, 1991). Contrary to dry sausages from western countries, meat starters are rarely used in preparing Chinese-style sausages. However, the quality of Chinese- style sausages could be improved by adding lactic acid bacteria (Huang, & Lin 1993). Some common ingredients for Chinese-style sausages are minced pork with slightly large particle size, high sugar content, rice wine, and spices (Lin, & Chao, 2001).

Lactic acid bacteria (LAB) are widely used in all type of dry fermented sausages to accelerate ripening time, improve color and flavor, inhibit undesirable pathogenic and spoilage bacteria by producing lactic acid and bacteriocin thereby improve the overall qua lity and shelf-life of products (Lewus *et al.*, 1991; Con & Gökalp, 2000; Erkkilä *et al.*, 2001, Sakhare, & Rao, 2003). So far, almost none of published report dealt with physicochemical and biochemical characteristics of dry fermented Chinese-style sausages is available. Therefore, this work was aimed to reveal the effects of LAB cultures namely *L. casei* subsp. *casei*-1.001 and *P. pentosaceus*-ATCC 33316 on the physico-chemical as well as biochemical qualities of dry fermented Chinese-style sausages.

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Methodology

Preparation of inoculums: Two strains of LAB: *L. casei* subsp. *casei*-1.001 and *P. pentosaceus*-ATCC 33316 obtained from China General Microbiological Culture Collection Centre, Beijing was sub-cultured in MRS broth medium for 3 days at 30^0C separately.

Cell pellets were harvested by centrifugation (Model HSC-20RA, Tumen, China) of respective broth at 10,000g for 15 min at 4°C, washed twice with 20mM phosphate buffer, pH 7.0, and finally resuspended in the same buffer (~10% of initial volume) (Fadda *et al.*, 2002). The number of bacterial cells in each suspension was adjusted to 10^7 cfu/g sausage batter at inoculation time by optical density method (Model WFZ-UV-2100, Unico™).

Sausage processing: Fresh boneless pork ham, backfat and all curing ingredients were procured from local supermarket. Large connective tissues and external fat were trimmed off with a sterile knife and then frozen overnight at about –10°C. Partially thawed meats were ground through a 9.5 mm plate meat mincer and carefully mixed with other curing ingredients as given in following proportion: pork lean (80 g), back fat (20 g), sugar (8.0 g), five spices powder (0.3 g), white pepper powder (0.1 g), common salt (2.0 g), MSG (0.5 g), sodium nitrite (0.012 g), sodium erythorbate (0.05 g), potassium sorbate (0.2 g), rice wine (1.0g), sodium tripolyphosphate (0.2 g) and chilled water (10 g). Three different batches of sausages were prepared by inoculating with LAB: P. pentosaceus-ATCC 33316 (CHS-PP), L. casei subsp. casei 1.001 (CHS-LC) and without starter (CHS-

CONT) as a control. After inoculation, batters were cured for 24 h at 4°C and then stuffed into edible collagen casing (3 cm in diameter, Nalo Faser, Germany), manually linked (10-12 cm long and 45 to 50 g in weight), fermented and ripened inside a laboratory ripening cabinet at 22°C (90-95% RH) for 3 days, first drying at 16±1°C (80-85% RH) for one week and last drying at 12±1°C (70-75% RH) for two weeks. Sampling was assigned as following: initially just before stuffing (0 day), after fermentation stage (3rd day), after one week of ripening (10th day), and final product (24th day). Five links of sausage samples were taken from each batch at a time. All analyses were done in triplicate.

Chemical analysis: The sausage samples were analyzed for moisture content, crude protein, crude fat, ash content and residual nitrite content by the method of AOAC (1997); total sugar content (as glucose) by DNS colorimetric method (James, 1995). Water activity (a_w) of sample was directly measured by water activity meter (Rotronic Hygroskop DT, Switzerland) after equilibrium at 25^{0}C. pH values by pH meter (Wang, 2000). Total volatile basic nitrogen (TVBN) from ground samples were extracted in 7% (w/v) trichloroacetic acid (TCA) using the method of Yin, & Jiang (2001) and then quantified by Conway Micro Diffusion Technique (Pearson, 1968). TBA (thiobarbituric acid) value for lipid oxidation was determined according to Vasavada, *et al.*, (2003). The non-protein nitrogen (NPN) fraction was extracted from 10g of sausages using 0.6N perchloric acid and then quantified by standard Kjeldahal method (Dierick *et al.*, 1974).

Physical analysis: Three links of sausage samples were brought to room temperature and each link was cut into halves. The color characteristics; *L* (lightness), *a* (redness) and *b* (yellowness), of each cross-section were measured by color difference meter (Model TC–PII G, Beijing Optical Instruments, China). The texture profiles *e.g.*, hardness, springiness, cohesiveness, and chewiness of sausage samples were determined according to Bourne, (1978); Lin, & Chao (2001). Three 1-cm sections from each link of sausages ere cut out and each section was compressed twice using a texture analyzer (Model TAXT2i, Stable Micro System, England). A 25 kg load cell and P0.5 adaptor with 2 mm/s test speed and 70% compression strain were applied.

Analysis of free amino acids: Free amino acids were extracted from the ground sample according to Aristoy and Toldrá, (1991) and pre-column amino acid derivatization was done by using OPA (*ortho*-phathalaldehyde) prior to analyze by high-performance liquid chromatography (HPLC) (Agilent 1100 Series, USA). The analysis column used was Hypersil ODS C_{18} (4 X 125) mm and the column temperature was 40^{0}C and flow rate 1.0ml/min. Peak identification and quantification was accomplished by determining the retention time and recoveries of amino acids standards (Sigma Chemical, St Louis, MO).

Analysis of biogenic amines: Two grams of finely ground and mixed dry sausage sample was mixed with 10 ml of 5% (w/v) TCA solution, carefully homogenized by Ultra Turex T18 for 5min and then filtered through 0.45µm filter. Taking 1 ml of filtrate into a centrifuge vial centrifuged at 10,000 rpm for 10 min. Finally, 0.5ml supernatant was transferred into glass vial for HPLC (Agilent 1100 Series, USA) analysis. Pre-column amine derivatization with OPA (*ortho*-phathalaldehyde) was done and the amines peaks were detected by using fluorescence detector at exciting wavelength 340nm and emission wavelength 450 nm. Standard amine samples e.g. Tyramine and Tryptamine were purchased from Sigma Aldrich Inc.3050 Spruce, Street St. Louis, USA and Histamine dihydrochloride from Fluka Chemical, GmbH CH-9471, Switzerland.

Data analysis: One-way analysis of variance (ANOVA) and Duncan's Multiple Range Test were applied to the triplicate data to compare the means at 95% of significance. Pearson's correlation coefficients were calculated whenever needed to show the significant correlations between two treatments. All statistical analyses were performed using the SPSS 11.5.0, (2002) statistical program.

Results and Discussion

Chemical Analysis

There were no significant differences ($p>0.05$) in fat content of dry sausages; however, protein, ash and sugar content were slightly varied among samples (Table 1). The sugar content was significant-ly ($p<0.05$) lower in LAB inoculated samples particularly *L. casei* subsp. *casei* 1.001. Higher lactic fermentation during processing of sausages as well as heterogeneous mixture of Chinese-style sausages due to larger meat and fat particle size could be attributed to such variations.

Table 1: Chemical composition of dry fermented Chinese-style sausages inoculated with LAB

Parameters	Sample		
(g/100g dm)	CHS-CONT	CHS-PP	CHS-LC
Protein	39.7 ^{ab} (1.33)	41.6^{b} (1.26)	38.7^{a} (1.17)
Fat	37.8 ^{a} (1.0)	36.4 ^{a} (0.9)	36.9^{a} (0.9)
Ash	7.1^{ab} (0.3)	6.7 ^{a} (0.2)	7.2 ^{b} (0.2)
Sugar	11.6^{ab} (0.61)	12.1^{b} (0.9)	10.8^{a} (0.32)

- Results are the mean of triplicates ± S.D.
- Different letters (a, b, c) in the same row are significantly (P=0.05) different

The changes in moisture and water activity during dry fermented Chinese-style sausages are shown in Figure 1.

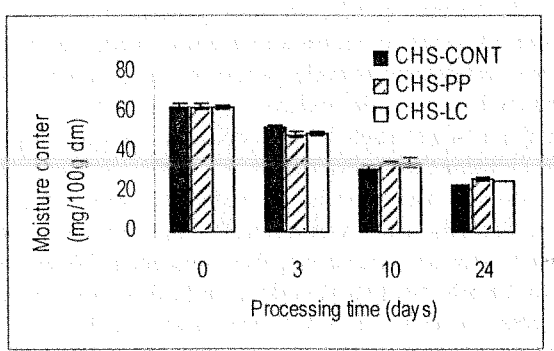

Figure 1: Changes in moisture content in dry fermented Chinese style sausages inoculated with LAB

The initial moisture content in sausages batters was approx 63%, which sharply decreased to around 26% at the end of ripening. The mild condition of two stages drying could significantly ($p<0.05$) reduce moisture by approximately 2.5 times. Initially, the a_w of sausage batters varied at range of 0.923 to 0.926 with out statistically significant ($p>0.05$) but at the end of ripening a_w significantly decreased with highest value (0.826) for control and lowest (0.803) for sample inoculated with *L. casei* (Figure 2). A significant correlation between moisture content and a_w ($r = 0.929$, $p<0.01$) clearly showed the dependency of aw on moisture content of dry sausages.

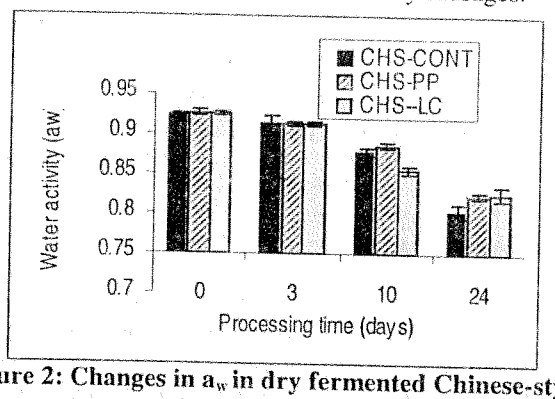

Figure 2: Changes in a_w in dry fermented Chinese-style sausages inoculated with LAB

The pH decline in samples added with LAB particularly *L. casei* subsp.*casei* 1.001 was very fast resulting the lowest pH value (4.23) in final product, whereas other two were statistically not significant ($p>0.05$) (Figure 3).

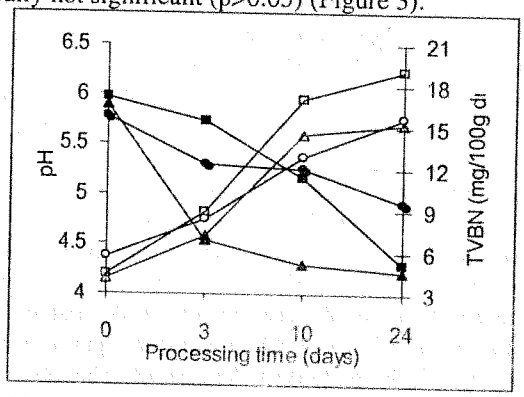

Figure 3: Changes in pH and TVBN values during ripening of dry fermented Chinese-style sausages inoculated with LAB.

[Solid: pH and Hollow: TVBN; Symbols:—▲—,—●—,—■—,inoculated with *P.pentosaceus*, *L. casei* subsp.*casei* and control without starter respectively.]

Likewise, initially TVBN values were at the range of 10.97 to 12.53mg/100g dm without statistically significant ($p>0.05$), which increased rapidly up to 10 days of processing and then started to decline in case of LAB treated samples ranging from 20.6 to 20.8 mg/100g dm, which were significantly ($p<0.05$) lower than the value obtained in control (25.7mg/100g dm) (Figure 3). Since, negative correlation ($r = -0.684$, $p<0.05$) between TVBN and pH of LAB treated samples during ripening could influence on the TVBN values as described by several authors (Yin, & Jiang 2001; Yin *et al.*, 2002), who have been reported the action of LAB producing lactic acid and bacteriocin thereby neutralize the TVBN values in fermented products. Like TVBN values, thiobarturic acid (TBA) values in dry fermented Chinese-style sausages found to be increased over ripening time (Figure 4).

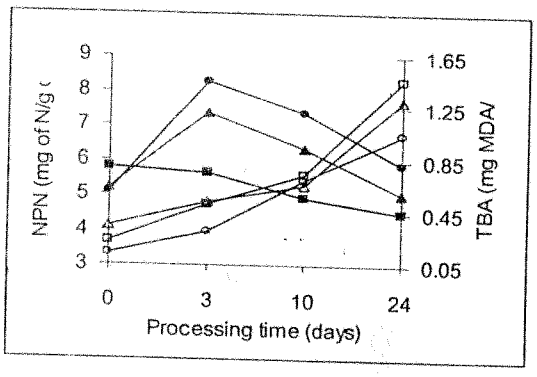

Figure 4: Changes in NPN and TBA values during ripening of dry fermented Chinese-style sausages inoculated with LAB. Solid: NPN and Hollow: TBA; Symbols: —▲—, —●—, —■—, inoculated with *P.pentosaceus*, *L. casei* subsp. *casei* and control without starter respectively.

The TBA values were gradually increased until 10 days of ripening after which more rapidly increased over 1.0 mg MDA/kg. The control sample had the highest TBA value (1.46mg MDA/kg), whereas *P. pentosaceus* inoculated sausage had the lowes (B) gMDA/kg) and sample inoculated with L. *casei* subsp.*c* l had 1.3mgMDA/kg. Since starters like *P. pentosaceus* could have a strong antioxidant activity upon unsaturated fatty acids, the TBA value for products added such organisms could be lower than in the naturally fermented (Talon, *et al.*, 2000). However, it is likely seemed that the bacterial lypolysis as well as higher fat content and ripening for long time could accelerate lipid oxidation in sausages

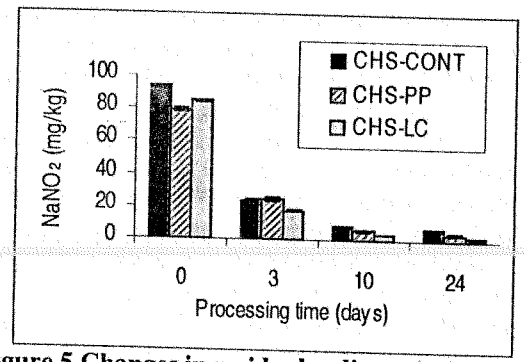

Figure 5 Changes in residual sodium nitrite in dry fermented Chinese-style sausages inoculated with LAB

Residual nitrite level in meat products is very important, as it can form different carcinogenic N-nitrosamines reacting with certain amino compounds (Sen *et al.*, 1974). Ripening of the sausages considerably decreased the nitrite level in sausages

(Figure 5). The lowest nitrite level (2.00mg/kg) was found in sample inoculated with LAB strain of *L. casei* subsp. *casei* 1.001, whilst the significantly highest ($p<0.05$) was found in control sample (7.10mg/kg). Lactic acid bacteria, because of their pH lowering capacity, are reported to dissipate residual nitrites in cured meats (Yin, & Jiang, 2001). The nitrite levels were below 5 mg/kg except in control, which were in general agreement with the results of other investigators (Meynier, *et al.*, 1999).

Physical Analysis

Effects on color: The effect of LAB on color characteristics of dry fermented Chinese-style sausages are given in Figure 6.

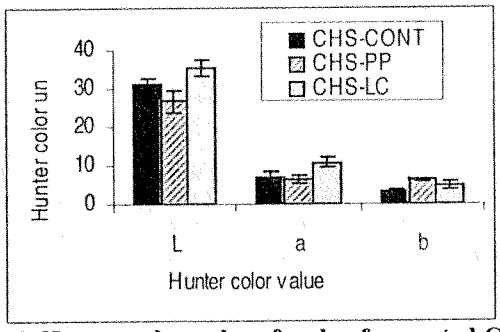

Figure 6: Hunter color values for dry fermented Chinese style sausages inoculated with LAB

Normally, the tendency for *L* value (lightness) and *b* value (yellowness) of products were decreasing over processing time, meanwhile very little changes in *a* value (redness) have been observed in the same sausage samples. The redness and lightness values for sausage inoculated with *L. casei* subsp.*casei* 1.001 were significantly higher ($p<0.05$), though other two samples were not significant in red color values.

Effects on texture: The texture profile analysis of dry fermented Chinese style sausages inoculated with LAB is presented in Table 2. From the results, no significant difference ($p>0.05$) have been found for springiness and cohesiveness of samples; however, the sausage inoculated with *P. pentosaceus*-ATCC33316 exhibited significantly lower ($p<0.05$) values for hardness and chewiness. The sausage treated with *L. casei* subsp.*casei* 1.001 showed significantly ($p<0.05$) higher value of hardness than in any other treatments, though no significant difference was found with control. A high amount of acidity (pH 4.23) produced by *L. casei* subsp. *casei* 1.001 in sausage could accelerate protein denaturation when it reaches an ioselectric pH for myofibrillar protein and then caused to rapid drying out resulting increased in hardness. Chinese-style sausages are usually reported as less juicy and tougher than other sausages (Lin, and Chao, 2001). Use of coarse ground type of lean meat and fat particle to prepare Chinese-style sausages could be attributed to a harder texture. The textural property of sausages also could be linked with a degree of proteolysis caused by dominant micro-flora during ripening (Herranz *et al.*, 2003).

Table 2: Texture profile analysis of dry fermented Chinese-style sausages inoculated with LAB

TPA parameters	Sausage sample		
	CHS-CONT	CHS-PP	CHS-LC
Hardness (g)	11121.5[b] (1533.7)	7419.0[a] (637.6)	13391.4[c] (868.3)
Springiness (mm)	0.884[a] (0.1)	0.879[a] (0.2)	0.83[a] (0.03)
Cohesiveness	0.599[a] (0.2)	0.53[a] (0.01)	0.534[a] (0.05)
Chewiness (g x mm)	5017.7[ab] (1045.2)	3476.6[a] (963.8)	5909.8[b] (92.3)

- Results are the mean of triplicates ± S.D.
- Different letters (a, b, c) in the same row are significantly (P=0.05) different

Biochemical characteristics

Effects on free amino acids: The glutamic acid, glycine, alanine, arginine, and proline (concentration greater than 30mg/100g dry matter), summing up approximately 81% of the total were the predominant free amino acids in the minced mix (Table 3). However, after ripening, the alanine was decreased markedly but other free amino acids such as valine, phenylalanine, leucine and lysine and even tryptophan, which was not detected in minced mix were widely increased in final products inoculated with LAB. In contrast, cysteine was only in trace amount excluding minced mix sample and the sample added with *P. pentosaceus*-ATCC 33316. The amino acids such as Glu, Gly, Arg, Val, Trp, Phe, Leu, Lys and Pro (concentration greater than 30mg/100g dry matter) were major free amino acids that summing up more than 88% of the total; however, in control Val, Phe and Lys concentration couldn't exceed to a level of 30mg/100 g of dry matter. The products CHS-PP added with *P. pentosaceus*- ATCC 33316 showed highest content of free amino acids (1228.2 mg/100g dm), whereas control had lowest. Furthermore, total free amino acids content were increased by ~61 to 65% in LAB inoculated ripened products, whilst only by ~23% in control. The result obtained was in general agreement to the result of Hughes, *et al.*, (2002), who have been reported that the starter cultures caused more production of free amino acids in dry fermented sausage as compare to control (no starter).Furthermore, free amino acids were significantly increased after ripening as reported by Beriain, *et al.*, (2000), Since free amino acids were highly correlated with flavor components in cured meat products (Cordoba, *et al.*, 1994), its abundance in dry fermented sausage seems to be beneficial to develop specific flavor components in dry fermented Chinese-style sausages.

Effect on biogenic amines formation: Biogenic amines are low molecular weight organic compounds formed mainly by decarboxylation of amino acids or by amination and transamination of aldehydes and ketones. Its presence in meat particularly fermented sausages is very common and high quantity could cause food poisoning outbreaks (Shalaby, 1996).

The control sample showed approximately 2 times more histamine and 1.2 times more tyramine than the sample inoculated with *P.pentosaceus*- ATCC 33316. Likewise, 2 times more histamine and 1.5 times more tyramine as compare to other sample inoculated with *L. casei* subspp.*casei* 1.001(Figure 4). It is clear that control sample (without starter) was found to be more contaminated with biogenic amines particularly histamine and tyramine (Fig 4). In contrast, pure cultures of LAB could have inhibitory effects on formation of biogenic amines, though no such effect on tryptamine was seen (Figure 7).

Table 3: Free amino acids in dry fermented Chinese style sausages inoculated with LAB

Free amino acids	Sample (mg/100 g of dry matter)[a]			
	Minced mix	CHS-CON	CHS-LC	CHS-PP
Glutamic acid	345.6	368.0	503.3	528.1
Serine	13.0	21.9	16.8	28.7
Histidine	17.1	20.9	22.77	14.9
Glycine	31.2	41.9	50.45	49.1
Threonine	12.3	23.4	28:3	26.8
Alanine	37.8	11.1	15.64	8.5
Arginine	191.9	163.1	224.83	214.1
Tyrosine	4.0	12.7	13.0	9.3
Cysteine	2.0	tr	tr	8.9
Valine	16.7	29.2	37.53	39.2
Methionine	3.3	11.7	16.82	17.4
Tryptophane	N.D.	69.6	93.48	81.4
Phenylalanine	13.1	26.9	35.72	39.2
Isoleucine	2.5	15.3	20.35	22.1
Leucine	8.3	31.6	42.77	45.8
Lysine	5.4	29.4	39.91	42.7
Proline	30.5	40.1	38.46	52.2
Total	743.7	916.8	1200.1	1228.2

[a] Minced mix sample had no culture that was just taken prior to stuffing into casing and the final products are 24 days of ripening. N.D. = Not Detected; tr = trace amount

Several authors have been reported that different biogenic amines at varied concentrations formed in different types of dry fermented sausages (Hernández-Jover, *et al*, 1996; Coïsson, *et al*., 2004). Along with the results of other authors, our result also confirmed that biogenic amines were highly accumulated in spontaneously fermented sausage and mainly caused by excessive growth of *Enterobacteriaceae and* amine contaminated natural lactic acid bacteria (microbiological results not shown) (Durlu-Özkaya, *et al*., 2001; Bover-Cid, *et al*., 2003). LAB particularly, *L. casei* subsp. *casei* 1.001 added samples showed an effective inhibitory action on biogenic amines due to its antagonistic characteristics against such spoilage bacteria.

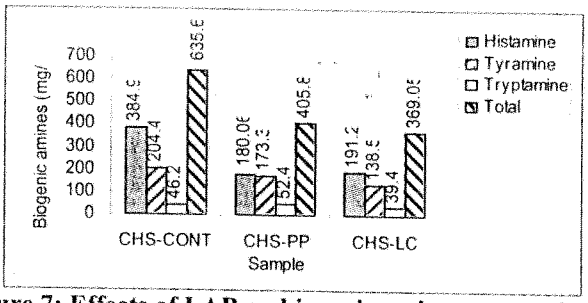

Figure 7: Effects of LAB on biogenic amines content in dry fermented Chinese-style sausages

Conclusions

LAB culture greatly influenced the physical, chemical as well as biochemical characteristics of dry fermented Chinese-style sausages resulting better quality products than the product produced by spontaneously fermented one.

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Influence of *Pediococcus pentaseous*, *Saccharomyces cerevisiae* and *Aspergillus niger* on Sensory, Biochemical and Nutritional Characteristics of *Masyaura* -A Legume-based Traditional Fermented Food of Nepal

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Masyaura, a legume based traditional fermented food product of Nepal, was prepared from blackgram dhal and colocosia tuber by inoculating Pediococcus pentasaceus, Saccharomyces cerevisiae and Aspergillus niger. Masyaura thus prepared was evaluated for sensory, biochemical and nutritional characteristics. The results revealed that Pediococcus pentosaceus and Saccharomyces cerevisiae resulted sensorily acceptable Masyaura but Aspergillus niger resulted unacceptable one. Nutritional quality of Pediococcus pentasaceus and Saccharomyces cerevisiae inoculated Masyaura was comparable to Masyaura prepared by traditional sun-drying technique. Sulphur amino acids were the first limiting amino acids in Masyaura.

Keywords: *Masyaura*, Culture inoculation, Sensory quality, Biochemical and nutritional evaluation, Fermented food, Nepal

Introduction

Masyaura is an important legume based fermented food of Nepal has been used by all classes of people as a substitute for meat (Karki, 1986). It is usually prepared in cottage or home scale level and used as an adjunct in curry. *Masyaura* is one of the famous savory foods used commonly by all class of people. The raw materials are legumes especially blackgram and greengram and vegetables like colocosia tuber, ashgourd, raddish etc. *Masyaura* technology is also a good method of preserving the perishable vegetables (Gajurel & Baidya, 1979). *Masyaura* is usually prepared by simultaneous natural fermentation during the sun drying for 3 to 5 days. Lactic acid bacteria have been recorded accounting more than 90% of the total flora. Direct involvement of lactic bacteria *Pediococcus Pentosaceus* in the traditional *Masyaura* preparation had been reported (Dahal, Rao & Swamylingappa, 2003). *Saccharomyces cerevisiae* in yeast and *Aspergillus niger* in mold was also recorded during the preparation of *Masyaura* as the dominant microflora. In this study an attempt was made to investigate the role of *Pediococcus pentosaceus*, *Saccharomyces cerevisiae* and *Aspergillus niger* on biochemical, physicochemical and sensory quality of the *Masyaura* since the information is lacking in the literature. The information obtained in this investigation would encourage and could lead the *Masyaura* technology in commercial scale.

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Materials and Methods

Raw materials: Blackgram (*Phaseolus mungo*) dhal was collected from local market of Kathmandu, Nepal and Colocosia (*Colocosia esculanta*) tuber was purchased from the local market of Wuxi, China.

Microbial Cultures: *Pediococcus pentosaceous* 1.2695 (ATCC 33316), *Saccharomyces cerevisiae* As. 2.399 and *Aspergillus niger* 3.4309 strains were collected from the China General Microbiological Culture Collection centre (CGMCCC), Beijing, P.R. China. Supplier's instruction was followed for the cultivation of the cultures. *Pediococcus pentasaceous* was grown in MRS medium, transferred to the sterile vial with 20% sterile glycerol and preserved at -70°C as the method given by Parton & Willis, 1990 and subcultured as per the method given by Valdez, 2001. Yeast and Molds were stored at 4° C.

Preparation of Masyaura

Preparation of dough: (a) Natural Dough: Cleaned split blackgram dhal was washed thoroughly with water to remove husk and foreign matter and soaked for 16h in water. The soaked dhal was lightly hand washed in tape water to remove husk and ground into a thick paste (Moisture around 70%) using a wet grinder. The colocosia tuber was washed peeled and finally wet grinded to get thick paste (Moisture of colocosia paste was maintained about 70% with colocosia powder. Colocosia tuber was sliced, blanched at 70°C for 5 min dried at 50°C and was grinded to get powder). Blackgram paste and Colocosia paste were mixed in a ratio of 1:1 (w/w) to get homogeneous dough. (b) Sterilized dough: Soaked, dehulled and drained blackgram dhal with water (1:1 w/v) and peeled colocosia tuber as such in two separate containers was sterilized at 121°C for 15 min and cooled to room temperature. The

sterilized blackgram dhal and colocosia tuber were crushed separately to get blackgram paste and colocosia paste respectively. They were mixed in the ratio of 1:1 and mixed homogeneously.

Culture Inoculation: (a) *Pediococcus Pentosaceus: Pediococcus Pentosaceus* was grown in MRS broth for 3 days at 37°C, centrifuged at 10,000 rpm at 4°C for 20 min. Supernant was discarded and the pellet was washed twice using phosphate buffer (20 mM, pH 7.0). Same buffer was used to dissolve the bacterial cells (20% of the original medium), vortexed. The number of cells in the suspension was determined using the linear standard curve (Standard curve was previously plotted after maintaining the OD_{540} of the same suspension at 0.2, 0.4, 0.6, 0.8, and 1.0 with the corresponding number of the cells in the respective suspension as determined by the plate count method after the appropriate dilution of the different set of suspensions. (b) *Saccharomyces cerevisiae*: *Saccharomyces cerevisiae* was grown on wort medium for 2 days at 30°C and centrifuged at 5,000 rpm for 20 min. Supernant was discarded and the pellet was dissolved in sterile physiological saline [0.9% (w/v) NaCl] using 20% of the original medium and vortexed. The suspension was used to count the yeast cells under microscope on Thoma counting chamber. (c) *Aspergillus Niger: Aspergillus niger* was grown on the agar slant following the supplier's instruction for 7 days at 27°C. Inoculum was prepared by flooding the mold with sterile physiological saline [0.9% (w/v) NaCl]. The mycelium was gently rubbed with a sterile spatula and the suspension was washed and centrifuged (5000 rpm, 15 min) twice. The pellet was resuspended in sterile physiological saline. The sporangiospore (further referred to as spore) count of the suspension was determined using a Thoma counting chamber. To activate the spores before inoculation, appropriate spore was transferred into a 20 ml sterile acidified (pH 4.0) of mold broth media (suppliers instruction), incubated for 6 h in a shaking water bath at 42°C, harvested by centrifugation (5000rpm, 15 min), washed and suspended in sterile physiological saline. Cultivation method was followed according to Noots et al., 2001. Inoculum size for *Pediococcus pentosaceus* was used at 10^7 cells/g of the dough. Inoculum size for both yeast and mold was 10^4/g separately on dough. Inoculum size was used on basic background of the natural dough. (Lactic counts of 7.4 log CFU/g and yeast and mold count of 3.7 logs CFU/g) (Dahal et al., 2003)

Dough distribution on tray: Both Natural and Sterilized dough inoculated with different microorganisms were mixed to get homogeneous dough and distributed on trays separately making spherical balls of about 20-30 gms and with a spacing of about 1 cm between the balls.

Fermentation: Trays were subjected for fermentation at 30° C. Trays were then removed after 24, 48 and 72 h of fermentation (Relative humidity: 80-90%).

Mechanical drying: Trays were then dried at 50°C for 12-16 h. The dried product was packed and stored in sealed polythene bags at ambient temperature. The dried *Masyaura* was powdered in a grinder to pass through 60-mesh sieve and used for chemical analysis.

Organoleptic Evaluation: Organoleptic evaluation of *Masyaura* samples was done using the 5-point Hedonic scale. *Masyaura* samples were boiled as traditional curry preparation and were subjected to the organoleptic evaluation. The 10 panels of semi-trained judges carried out the sensory evaluation following the criteria as follows.

Excellent:	Pleasant smell, Pleasant taste with good favor, chewy texture, no bitterness .....5.
Very good:	Good sour and pleasant taste with flavor, good texture less bitterness.....4.
Good:	Moderate flavor, moderate taste, moderate texture and moderate sour.....3.
Average:	Less favor, less taste, less soft texture and less sour.....2
Poor:	Putrefaction flavor, off smell, soft texture and bitter taste.....1

Acidity and pH: Total acidity was calculated in terms of lactic acid by titrating against 0.1N Sodium hydroxide according to the AOAC (1990) method; pH was measured directly using a digital pH meter (Leici Instrument Company, Shanghai, P. R. China). *Proximate composition*: Moisture, protein ash fat and fiber were estimated by the standard AOAC (1990) method. *Free sugar and reducing sugar*: Free sugar in the defatted sample was extracted by 70% ethanol until the residue was sugar free. Total carbohydrates and reducing sugar in the extract were estimated respectively by the phenol-sulphuric acid method of Dubois et al (1956) and DNSA method (Miller, 1959). *Soluble protein and amino nitrogen*: Soluble protein was determined according to the method of Mattil (1971); soluble protein in supernant was estimated by Kjeldahl method using a factor of 6.25. Amino nitrogen was determined by the modified Serenson method as described in AACC (1962). *Minerals*: The samples were dry-ashed and ash solution was used for estimation of minerals. Phosphorous was estimated colorimetrically (Ranganna, 1995). Calcium, Iron, magnesium, potassium, cupper, manganese and Zinc were estimated in Atomic absorption spectrophotometer (Varian Company, USA). *Vitamins:* Thiamine, Riboflavin, Pyridoxine and Niacin were determined by HPLC method of Sarker et al., 1998. Derivatization of thiamine to thiochrome was done according to the method of Mickelsen et al., 1945. Vitamin B12 was determined according to HPLC method of Hudson et al., 1984. *Amino acid analysis*: Amino acid analysis was done according to HPLC method of Godel et. al. (1995). Tryptophan was estimated by the ninhydrin method of Swakais and Pest (1990) after extracting protein according to the method of Concon (1975). *Chemical score*: Chemical score was calculated using equation given below according to the method of Block & Mitchel (1946)

$$\text{Chemical score} = \frac{\text{Gms of EAA in test protein} \times \text{Gms of total EAA in egg} \times 100}{\text{Gms Total EAA in test protein} \times \text{Gms EAA in egg}}$$

In Vitro digestibility of protein and starch: The in vitro protein digestibility was determined by the method of Akeson & Stahman (1964) using pepsin and pancreatin enzymes. Starch

digestibility was determined by the procedure of Singh et al (1982) using pancreatic α-amylase, the released sugars were estimated by DNSA method as glucose (Miller, 1959). Starch was analyzed as glucoamylase method of AACC-1999 with subsequent measurement of glucose by DNSA method.

Results and Discussion

Masyaura was prepared by the inoculation of *Pediococcus pentasaceus*, *Saccharomyces cerevisiae* and *Aspergillus niger* cultures separately (Details in Materials and Method section). Sensory evaluation of *Masyaura* was carried out and the result is presented in Table 1. Sensory evaluation of *Masyaura* prepared by inoculation of *Pediococcus pentasaceus* resulted that there was not significant difference (P=0.05) for the overall sensory scores between control and the *Masyaura* inoculated and fermented for 24h. But *Masyaura* fermented for 48 and 72 h was found to be slightly inferior among them. Sensory evaluation for *Masyaura* prepared by inoculation with *Pediococcus pentasaceus* on sterilized dough resulted that non-inoculated control had lower sensory scores. Scores for the bacteria inoculated on sterilized dough was also lower below 3 (5-highest score). Sensory scores for *Masyaura* prepared by inoculation with *Saccharomyces cerevisiae* on natural dough resulted that yeast inoculated samples for 24h and 48h was superior to the control. The result indicates that yeast is the desirable microorganism for the acceptability of *Masyaura*. In case of *Saccharomyces cerevisiae* inoculated *Masyaura* on sterilized dough, the sensory scores were lower (almost less than 2). This indicates that only *Saccharomyces cerevisiae* might not result the acceptable *Masyaura*. In case of *Aspergillus niger* inoculation on natural dough, the sensory scores were very low i.e. less than 2 for all fermented *Masyaura*. The scores were significantly inferior to the control (non-inoculated fermentation for 24h) as indicated in Table 1.

Table 1: Mean panel score of *Masyaura* fermented with different microorganisms

Samples	*P. pentosaceous*	*S. cerevisiae*	*A. niger*
	Natural Dough		
Control, 24h*	3.8^{c}	3.3^{b}	3.7^{c}
Inoculated, 24h	3.7^{c}	3.9^{c}	1.4^{a}
Inoculated, 48h	3.2^{b}	3.8^{c}	2.1^{b}
Inoculated, 72h	2.5^{a}	1.8^{a}	1.3^{a}
	Sterilized Dough		
Control, 24h	1.7^{a}	1.9^{a}	2^{a}
Control, 48h	1.6^{a}	-	-
Control, 72h	2.1^{a}	-	-
Inoculated, 24h	2.9^{b}	2.4^{a}	1.4^{a}
Inoculated, 48h	3.2^{b}	2.1^{a}	1.5^{a}
Inoculated, 72h	2.0^{a}	1.6^{a}	1.4^{a}

- *- Best scored among uninoculated control
- [Sensory score 1-Lowest, 5-Highest]
- Different letters (a, b, c) in the same column are significantly (P=0.05) different

Similarly *Aspergillus niger* inoculation on sterilized dough, all samples were sensorily inferior and not significantly different to each other. This indicates that mold might be the undesirable microflora for the acceptability of the *Masyaura* product.

Mixed culture of Lactic acid bacteria and yeast could result better sensory scored *Masyaura* product. It has been reported that the use of the *Pediococcus* strains as starter cultures affords a standardized microbial inoculum to each batch regardless of the inherent microflora. The inoculum level of the starter culture (recommended at 10^{6} to 10^{7} microorganisms) had been recommended to provide the desirable bacteria at such a high level that is generally precludes the development of any chance contaminations and any undesirable effects due to their growth and metabolism during sausage fermentation (Bacus & Brown-1985).

Based on the sensory characteristics, one best scored *Pediococcus pentasaceus* inoculated *Masyaura* sample (30°C/24h/80-90%RH) and another *Saccharomyces cerevisiae* inoculated best scored sample (30°C/24h/80-90%RH) were selected. Biochemical composition of *Masyaura* is presented in Table 2. Bacteria inoculated samples showed higher amount of acidity value and sun-dried *Masyaura* showed lower amounts of acidity.

Table 2: Biochemical parameters of *Masyaura* fermented by different methods

Parameters/ samples	Sun-dried *Masyaura**	Controlled Fermented *Masyaura*	Bacteria Inoculated *Masyaura*	Yeast Inoculated *Masyaura*
Acidity (%) as Lactic	1.82 ± 0.05	2.39 ± 0.12	3.23 ± 0.29	2.28 ± 0.03
pH	5.1 ± 0.01	4.9 ± 0.01	4.6 ± 0.04	5.5 ± 0.02
Free sugar (%)	5.3 ± 0.37	6.8 ± 0.34	6.6 ± 0.49	6.8 ± 1.0
Reducing Sugar (%)	0.9 ± 0.15	1.9± 0.06	0.8 ± 0.09	1.5 ± 0.2

- Results are the mean of the duplicates ± S.D and are on dry basis.
- *Masyaura* prepared by traditional sun drying [5 days (R.T. 20°C, RH: 70%)] technique

Chemical composition *Masyaura* prepared by different method is presented in Table 3. Chemical composition revealed that *Masyaura* prepared by different methods are comparable to each other. Chemical composition also indicated that the overall B group vitamin in controlled fermented, bacteria inoculated and yeast inoculated *Masyaura* especially thiamine and riboflavin are higher to sun-dried *Masyaura* (Table 3) but Vitamin B12 in bacteria and yeast inoculated sample was little low. This may be due to the variation in fermentation conditions and due to the activity of different microorganisms to produce or consume various kinds of vitamins. The vitamin content of various fermented foods has been reported to be greater than that of the unfermented substrates. Niacin and Riboflavin contents were reported to be increased after *Tempeh* fermentation (Steinkraus, 1983).

Table 3: Chemical composition of *Masyaura* fermented by different methods

Parameters	Sun-dried *Masyaura*	Controlled Fermented *Masyaura*	Bacteria inoculated *Masyaura*	Yeast inoculated *Masyaura*
Moisture (%)	9.7 ± 0.56	6.9 ± 0.13	6.8 ± 0 .21	7.2 ± 0.15
Protein % (N2×6.25)	22.3 ± 0.72	21.9 ± 0.03	21.4 ± 1.02	22.4 ± 0.65
Ash (%)	4.9 ± 0.03	5.1 ± 0.11	5.0 ± 0.21	5.2 ± 0.27
Fat %	0.6 ± 0.06	0.6 ± 0.11	0.5 ± 0.06	0.6 ± 0.11
Fiber %	1.9 ± 0.32	1.7 ± 0.32	1.7 ± 0.12	1.7 ± 0.15
Carbohydrate % by diff	60.6	63.8	64.7	63.0
Calcium (mg/100g) *	168 ± 1.4	128 ± 1.1	121.5 ± 1.3	121.9 ± 0.71
Phosphorous (mg/100g) *	267.8 ± 5.3	268.5 ± 5.5	248.7 ± 7.6	242.9 ± 3.7
Fe (mg/100g) *	7.5 ± 0.01	9.4 ± 0.09	6.33 ± 0.12	5.82 ± 0.08
Magnesium (mg/100g) *	151 ± 0.4	153 ± 2	143.9 ± 2.3	143.2 ± 3.5
Potassium (mg/100g) *	1887 ± 6	2166 ± 4	1355 ± 7.7	1286 ± 11.2
Cupper (mg/100g) *	1.84 ± 0.01	1.75 ± 0.01	1.57 ± 0.04	1.35 ± 0.02
Manganese (mg/100g) *	1.62 ± 0.04	1.47 ± 0.01	2.11 ± 0.02	2.14 ± 0.03
Zinc (mg/100g) *	3.35 ± 0.04	3.13 ± 0.01	3.90 ± 0.02	3.68 ± 0.1
Thiamine (μg/100g) *	174 ± 9	609 ± 8	244 ± 7	358 ± 10
Riboflavin (μg/100g) *	78 ± 7	140 ± 1	115 ± 6	120 ± 6
Pyridoxine (μg/100g) *	4771 ± 59	3170 ± 65	3168 ± 67	3267 ± 67
Niacin (μg/100g) *	14377± 397	13997 ± 40	12388 ± 236	9882 ± 249
Vitamin B12 (μg/100g) *	2477 ± 53	1990 ± 36	565 ± 18	662 ± 5

- Results are mean of duplicates ± S.D.
- * Minerals and vitamins are on dry basis.

Amino acid composition of *Masyaura* is reported in Table 4. The essential amino acids for *Masyaura* prepared by different fermentation methods are comparable to FAO pattern (FAO, 1968) except for sulphur containing amino acids, which are deficient in *Masyaura* and in its ingredients. Total essential amino acid of sun dried, controlled fermented, bacteria inoculated and yeast inoculated *Masyaura* were 37.2, 31.7, 38.3 and 37.5 % of amino acids (Table 4) and total free essential amino acids were 5.1, 9.2, 10.8 and 13.1 % of free amino acids respectively (Table 5). Protein score calculated based on the essential amino acid composition was 40.9, 35.8, 43.0 and 41.0 % for sun dried, controlled fermented, bacteria inoculated and yeast inoculated *Masyaura* respectively (Table 6). Sulphur amino acids were the first limiting amino acids in all cases. In vitro digestibility of starch and protein in *Masyaura* are better than raw ingredients and are comparable to each other (Table 7).

The increased digestibility in *Masyaura* samples as compared to its raw ingredients may be due to fermentation and breakdown of starch and protein to lower molecular weight components and also due to the reduction in phytate levels resulting in the production of flavor and aroma as well as elimination of unfavorable compounds such as flatulence causing oligosaccharides, including raffinose and stachyose (Ebine 1989, Lopez 1992, Soni & Sandhu 1990, Urooj & Puttraj 1994, Siegel & Fawcett 1976, Wang & Hesseltine, 1981).

Table 4: Amino acid composition of *Masyaura* fermented by different methods

Amino acid	Sun-dried *Masyaura*	Controlled fermented *Masyaura*	Bacteria inoculated *Masyaura*	Yeast inoculated *Masyaura*
Aspartic acid	14.4	11.8	12.3	11.8
Glutamic acid	15.9	12.8	14.7	14.1
Serine	5.8	4.4	5.4	5.1
Histidine	2.5	2.1	2.6	2.5
Glycine	3.8	3.2	3.9	3.8
Threonine*	3.8	3.2	3.8	3.6
Alanine	4.4	3.5	4.6	4.4
Arginine	6.4	5.5	5.5	5.6
Tyrosine*	3.0	2.3	2.5	2.6
Valine*	4.2	3.9	5.1	5.2
Methionine*	0.9	0.7	1.3	1.3
Cystine*	0.8	0.6	0.6	0.5
Phenylalanine*	6.1	5.0	6.0	5.8
Isoleucine*	3.4	3.1	3.9	4.2
Leucine*	8.3	7.3	8.5	8.1
Lysine*	5.7	4.5	5.6	5.4
Proline	4.2	3.7	3.8	3.2
Tryptophan*#	1.0	1.0	0.8	0.9
% Total EAA	37.2	31.7	38.3	37.5

- Results are on g/100g protein and mean of duplicates.
- # Tryptophan determined by ninhydrin method * - Indicates the essential amino acid.

Table 5: Free amino acid composition of *Masyaura* fermented by different methods

Amino acid	Sun-dried *Masyaura*	Controlled fermented *Masyaura*	Bacteria inoculated *Masyaura*	Yeast inoculated *Masyaura*
Aspartic acid	1.3 (9.1)	1.6 (13.6)	2.0 (16.2)	1.90 (16.2)
Glutamic acid	0.6 (3.7)	0.8 (6.4)	1.3 (8.8)	1.23 (8.7)
Serine	0.1 (2.1)	0.3 (6.8)	0.3 (5.6)	0.31(6.0)
Histidine	0.1 (3.6)	0.1(5.8)	0.1 (5.0)	0.21 (8.5)
Glycine	0.1 (2.1)	0.2 (6.4)	0.3 (6.7)	0.23 (6.0)
Threonine*	0.2 (5.5)	0.3 (8.8)	0.4 (10.2)	0.31 (8.7)
Alanine	0.3 (6.4)	0.1 (2.6)	0.1 (2.4)	0.09 (2.0)
Arginine	0.5 (7.6)	0.9 (15.6)	1.1 (19.3)	1.17 (20.9)
Tyrosine*	0.2 (7.7)	0.2 (10.4)	0.5 (18.3)	1.05 (40.9)
Valine*	0.5 (11.0)	0.5 (12.9)	0.7 (13.3)	0.79 (15.2)
Methionine*	0.2 (19.2)	0.2 (21.7)	0.2 (13.4)	0.28 (21.7)
Cystine*	ND	ND	ND	ND
Phenylalanine*	0.2 (3.1)	0.4 (7.4)	0.5 (8.1)	0.50 (8.7)
Isoleucine*	0.1(3.9)	0.3 (8.7)	0.4 (9.8)	0.40 (9.5)
Leucine*	0.2 (2.5)	0.5 (7.1)	0.8 (9.9)	0.64 (7.9)
Lysine*	0.1 (1.6)	0.3 (5.5)	0.3 (5.7)	0.67 (12.5)
Proline	0.1 (1.9)	0.3 (7.1)	1.9 (48.6)	0.27 (8.5)
Tryptophan*,#	0.2 (19.4)	0.3 (33.7)	0.4 (43.2)	0.27 (30.7)
% Total free EAA	1.9 (5.1)	2.9 (9.2)	4.1 (10.8)	4.91 (13.1)

- Results are on g/100g protein and expressed as mean of duplicates.
- Values in parenthesis indicate % free amino acids.
- # Tryptophan determined by ninhydrin method.
- * - Indicates the essential amino acid.

ND - Not detected.

Table 6: Chemical Score* of *Masyaura* fermented by different methods

Essential Amino Acids.		Sun-dried *Masyaura*	Controlled fermented *Masyaura*	Bacteria inoculated *Masyaura*	Yeast inoculated *Masyaura*
Lysine		117.1	110.1	113.4	110.3
Threonine		116.3	114.3	112.8	108.1
Valine		85.2	94.5	100.1	105.1
Leucine		134.3	138.7	133.9	130.7
Isoleucine		62.9	67.8	71.6	78.0
Methionine + Cysteine		40.9	35.8	43.0	41.0
Tryptophan		102.5	114.4	78.2	86.8
Phenyl alanine + Tyrosine		124.8	118.0	114.7	114.5
Protein Score		40.9	35.8	43.0	41.0
Limiting amino acids	1st	**Sulphur**	**Sulphur**	**Sulphur**	**Sulphur**
	2nd	Isoleucine	Isoleucine	Isoleucine	Isoleucine
	3rd	Valine	Tryptophan	Tryptophan	Valine

* Values calculated considering the amino acid composition of egg protein as the reference.

Table 7: In Vitro starch and protein digestibility of *Masyaura* fermented by different methods

Sample/Digestibility	Starch Digestibility	Protein Digestibility
Blackgram dhal	72.3 ± 0.28	83.9 ± 4.11
Colocosia tuber	49.9 ± 0.58	79.0 ± 1.36
Sun-dried *Masyaura*	71.0 ± 0.96	91.2 ± 0.21
Controlled fermented *Masyaura*	78.8 ± 1.48	90.1± 0.80
Bacteria inoculated *Masyaura*	86.9 ± 3.8	85.3 ± 1.9
Yeast inoculated *Masyaura*	84.7 ± 2.5	96.9 ± 0.6

• Results are the mean of duplicates ± S.D.

Conclusions

In conclusion, lactic acid bacteria and yeast seemed to be the responsible microflora for the sensory characteristics of *Masyaura* product. Biochemical and Nutritional quality of controlled fermented *Masyaura* were comparable to the *Masyaura* prepared by traditional sun-drying technique. Further works on influence of mixed cultures are encouraged.

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Preparation of Carrot Candy and Study on its Quality Parameters

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Carrots were washed, scrapped off thin outer layer, cut into uniform size, pricked, and blanched; and candy was prepared by slow and fast process. The candying process was carried out by varying citric acid content from 0 to 0.3% in syrup. The different quality parameters and chemical composition were analyzed. The rate of sugar uptake was found initially to be increased at a faster rate and then slowly during later period. Candy prepared in syrup containing 0.3% citric acid was found to be sensorily superior with regard to color, flavor, taste, and texture. The candy prepared from fast process was sensorily preferred than slow process.

Keywords: Carrot, Candy, Quality parameters, Sensory evaluation

Introduction

Candy is attractive and palatable product for all aged people, which is prepared by replacing the moisture content of fruit by penetration of sugar during candying (Jackson, et al 1985). Candying is one of the oldest method of preservation of food and antidotes the manufacture of refined sugar. Preservation of candy is by dehydration of fruit by osmotic pressure of the sugar solution (Woodroof et al., 1985) with the water activity lower than that of fruit (Karel, 1976). The preserve, which is dried lastly, reduces the moisture content and plays role in preservation giving it a better texture. The acid added for its inversion also provides better taste. β-carotene of carrot is changed into vitamin A when ingested into the body. When cooked the amount and absorption of β-carotene increases and carrot becomes more digestible (Dewan, 1994). The present work was undertaken to prepare carrot candy from carrot and also to optimize the acid content during syruping to produce better taste and color in the product.

Materials and Methods

Candy making process

Preliminary processing operation: Carrot were washed with clean water and trimmed crown and tail, and then scrapped off the outer thin layers. The scrapped carrots were cut into Uniform Square or rectangular shaped piece, each piece having about 3-5gm weight and 1.5- 2cm in size. The carrot pieces were then pricked all around it with the help of stainless steel pins to facilitate the transfer of moisture, sugar syrup, etc. The pricked carrot pieces were blanched in plain hot water at temperature of 70-75°C for 17-20 minutes until carrot pieces become soft.

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Candying process: (a) Slow process. The sugar syrup of 30°Bx was prepared & required concentration of citric acid was used. The sugar was added on alternate days to increase the concentration of syrup by 10°Bx until the concentration of syrup reaches 60°Bx. Then the TSS was raised by 5°Bx on alternate days till the final syrup TSS reaches 75°Bx. Then the carrot pieces were left as such for about 7 days to bring into equilibrium. (b) Fast Process: The carrot pieces were boiled in sugar syrup of 30°Bx and then boiling continued till the final concentration of syrup reaches75°x. Thus, syrup must be of sufficient amount such that the pieces remains immersed even when concentration of syrup reaches 75°Bx and then cooking was continued with frequent stirring until TSS reaches 75°Bx.

Packaging and storage: The prepared candy were packed in polyethylene packaging materials, sealed and stored at ambient condition.

In slow process, the concentration of sugar in syrup was raised by 10°Bx, boiled for 4min and left for one day likewise in each successive day; this process was repeated until the concentration was reached to 75°Bx. At 75°Bx the pieces were left for one week and again left for three days in syrup containing 01% citric acid. In each successive day the sample were taken for analysis of sugar uptake. In fast process, the carrot pieces were cooked in 30°Bx and cooking was continued until final concentration of syrup reached to 75°Bx by adjusting the amount of syrup such that the pieces remain immersed even when the concentration reached to 75°Bx. The pieces prepared by either of the above method were strained, washed under running tap water, dried in dryer at 65°C for 10hrs and packed in polythene pouch and stored in cool and dry place. The protocol for the preparation of carrot candy is given in Figure 1.

Sensory Evaluation: The five samples coded with A, B, C, D, E were given to 10 panelists for evaluation of quality attributes like color, taste, flavor and texture. The panelists

were asked to rate the products on Hedonic scale and rank the products according to their quality.

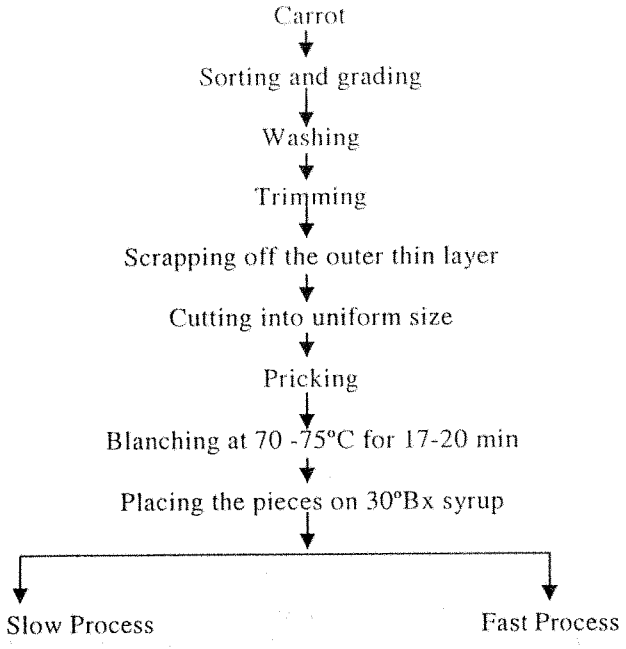

Figure 1: Flow Chart of Carrot Candy Preparation

Analytical methods: Symbols A, B, C, D were given to the candy prepared by fast process containing 0, 0.1, 0.2, and 0.3% citric acid respectively in sucrose syrup. Symbol E was referred to the candy prepared by slow process and containing 0.1% citric acid in sucrose syrup. The fresh carrot and candy samples were analyzed for moisture, titrable acidity, sugars and total soluble solid. The above determinations were carried out by following the procedure given in Rangana (1994).

Results and discussion:

Carrot candy was prepared varying the concentrations of citric acid after the successive operation of washing, pricking, blanching, candying, draining and drying. The candy was prepared by using the same sugar i.e., sucrose and from carrot prices, which were blanched to make it soft but varying the acid concentration of 0%,0.1%, 0.2%, and 0.3% using citric acid. This was done in fast process while in slow process; there was only one sample with 0.1% citric acid. The chemical composition of candy, sugar uptake of both fresh carrot and prepared candy were determined. The best carrot candy was found out from sensory evaluation. The chemical composition of the fresh carrot is given in Table 1.

Table 1: Chemical composition of carrot

Constituents (g/100gDM)	Fresh carrot
Crude protein	1.1
Ash	0.8
Fat	Trace
Sugar (total)	4.5
Dry matter	11.8
Crude fibre	1.0

- DM-Dry matter

Table 2: Chemical composition of carrot candies

Constituent	A	B	C	D	E
Moisture%	14.0	13.5	14.5	15.4	14.0
Reducing sugar%	Neg.	23.1	25.0	28.9	24.8
Total sugar%(as reducing)	72.1	71.3	72.7	73.1	71.4
Non-reducing sugar	72.1	48.1	47.6	44.1	46.6
Sucrose%	68.5	45.7	45.2	41.8	44.3
Total sugar%	68.5	68.9	70.3	70.9	69.1
Acidity%	0.03	0.28	0.33	0.45	0.30

Table 2 indicates that as citric acid content increases more inversion occurs and thus sucrose (non-reducing) converted into invert sugar (reducing), so the reducing sugar content increases with the increase in acid. The candy made by cooking in sucrose solution without acid had no reducing sugar or in negligible amount. Sucrose, glucose syrup and glucose play a prominent part in candy making. From several literatures, it was found that addition of acid during candying causes inversion of sucrose into invert sugar that prevents from crystallization of sucrose. The high content of invert sugar results in softer texture and sticky nature of the product. The sucrose content was found to be highest for candying with no acid content and was found to be decreasing in along with increasing acid content. It was found to be least with the one with highest amount of acid i.e. 0.3% citric acid content. The sucrose content for samples prepared with similar syrup showed higher in slow process than in fast process. On candying by fast process and slow process with different sugar concentrations of citric acid on syrup, the rate of sugar uptake of carrot pieces at different sugar concentrations starting from 40, 50, 60, 65, 70 and 75°Bx were determined (Figure 2). The initial sugar uptake was higher for the sample cooked only in sucrose syrup and went on decreasing with increasing acid content. The initial sugar uptake in candy containing same syrup i.e.0.1% citric acid in sucrose solution was found to be higher in slow process than in fast process. Initially the sugar uptake rate was higher than in final stage for all samples. The slow uptake in final stage was low due to viscous ness of the syrup. The %sugar uptake at final stage was found to be highest for sample D that may be due to higher inversion of sucrose by high acid and easy for uptake of sugar.

Among different samples prepared syrups containing 0, 0.1, 0.2, 0.3% citric acid, the carrot candy prepared by cooking in sugar syrup containing 0.3% citric acid was found to be preferred by panelists on sensory evaluation. The sensory evaluation of the different five products prepared was carried out by employing panelists on the quality attributes, color, flavor, taste, and texture. The graphical representation of the mean sensory score is shown in Figure 3.

The result of sensory evaluation showed that sample D was found to be the best in colour, flavour, texture and taste. However, the texture was softer in comparison to other samples and was slightly sticky. The sample E was found to be least desirable. Sample B and C were found to be equal. There was not much significant different ($p<0.05$) among sample in flavor. However, some flavor loss had occurred in product prepared with slow process. This might be due to longer time of cooking. The taste of the products prepared in syrup containing 0.1% citric acid was equally preferred when processed either by slow or fast process.

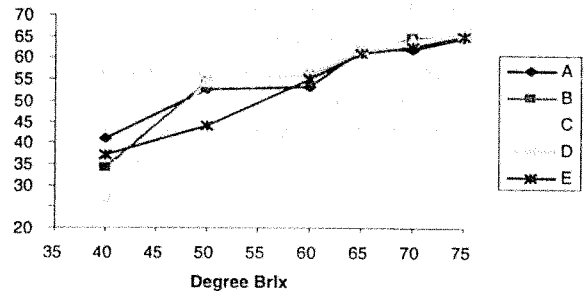

Figure 2: Rate of sugar uptake by carrot pieces at different sugar concentrations

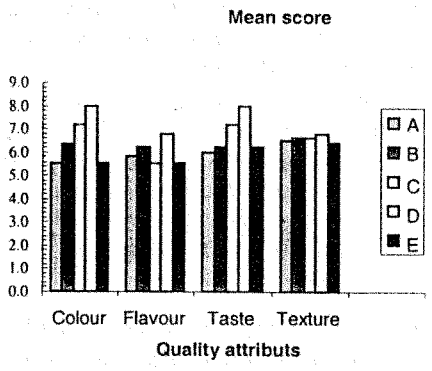

Figure 3: Graphical representation of mean score of sensory evaluation

Conclusions

Carrot candy can be prepared from locally available raw materials by simple method of preparation. The carrot candy prepared by fast process in syrup containing upto 0.3% citric acid was sensorily acceptable.

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Preservation of Strained *Jand* by Pasteurization

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Jand, a popular cereal-based alcoholic beverage indigenous to Nepal, has a very short shelf-life, of the order of 2-3 hours, after it has been strained. Trial preservation of the strained jand by pasteurization showed encouraging results. The time-temperature combination of 75°C/15 min for the pasteurization was arrived at from a separate trial. The changes in physicochemical properties (pH, acidity, reducing sugar and alcohol content) and sensory quality (flavor, color and homogeneity) of two treatments, viz., pasteurized and preserved at 10°C, and pasteurized and preserved at ambient temperature (20-25°C), were compared with control (freshly prepared and pasteurized jand) periodically at an interval of 12 days for a period up to 48 days of preservation. The treatments and the control showed no significant difference ($p<0.05$) in terms of physicochemical properties with storage time. Strangely though, treatments were significantly superior to control ($p>0.05$) in terms of flavor. The sensory qualities of the two treatments were not significantly different. The present study shows that the shelf-life of Jand can be extended to at least 48 days by a simple pasteurization treatment.

Keywords: Pasteurization, Physicochemical properties, Sensory quality, Shelflife, *Jand*, Nepal

Introduction

Jand is a traditional, undistilled alcoholoic beverage produced by solid-state fermentation of starchy cereals, notably fingermillet (*Eleusine coracana* L. Gaertn). The fermentation is initiated by a mixed culture called *murcha* (Karki, 1986), which consists of yeasts (species of *Saccharomyces*, *Hansenula*), molds (species of *Aspergillus* and *Mucor*) and lactic acid bacteria (species of *Lactobacillus*).

In Nepal, fingermillet is the most preferred substrate material for *jand* preparation. Fingermillet is considered to yield *jand* of unmatched sensory quality. The basic reason behind it is the uniqueness in flavor of *jand*. The technology of *jand* making has been known since antiquity (Rai, 1991). It is unfortunate that the same primitive traditional technology prevails to this day. In spite of improvement in our understanding on science related to traditional fermentations, nothing has much changed with time. On the other hand, many similar traditional technologies in other parts of the world have met with tremendous success, African sorghum beer (Novellie and Schaepdrijver, 1986), Japanese sake (Dasilva, Dommergues, Nyns & Rattledge, 1987), to name a few. The major setback in the commercialization of *jand* is the lack of research and development on it. To date, *jand* has not been able to receive the share of attention it so well deserves. Another hurdle in its popularization is the social stigma attached to it.

Jand is served in various forms, the most popular being strained *jand* (cloudy-white, aqueous extract).

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Strained *jand* has very short shelf-life, normally of the order of 2-3 hours. This could be one reason why *jand* has never been able to find a place on the shelves of stores. At this juncture, it is necessary that some simple alternatives be devised/developed for the preservation of strained *jand*. Theoretically, several possible treatments are possible, carbonation, sulfur dioxide addition and heat treatment. Given the complex nature of the product, suitability of any method will of course depend on many factors, the unraveling of which may require a very detailed and serious study.

The present study is an attempt to address the problem of shelf-life of strained *Jand* by adopting the simple option, namely, heat treatment. The study was aimed at developing a simple, readily affordable process adaptable at the village level. This paper addresses only the technological process developed in this study. Microbiological aspects will be presented in a later paper.

Materials and Methods

Materials: Optimally fermented (18 days old) millet *jand* (mash) was bought in bulk from a noted *jand* producer and divided into three lots, *viz.*, for Control, Trial Test, and Final Test. The lot meant for control was kept under refrigeration (–4°C) throughout the study period to control the microbial activity of yeast(s) and other microorganisms.

Millet was chosen for the present study because it yields *jand* of the best quality. Also, because millet is regarded as poor man's cereal, work on its value-addition, e.g., by commercializing *jand*, has a bright scope in Nepal.

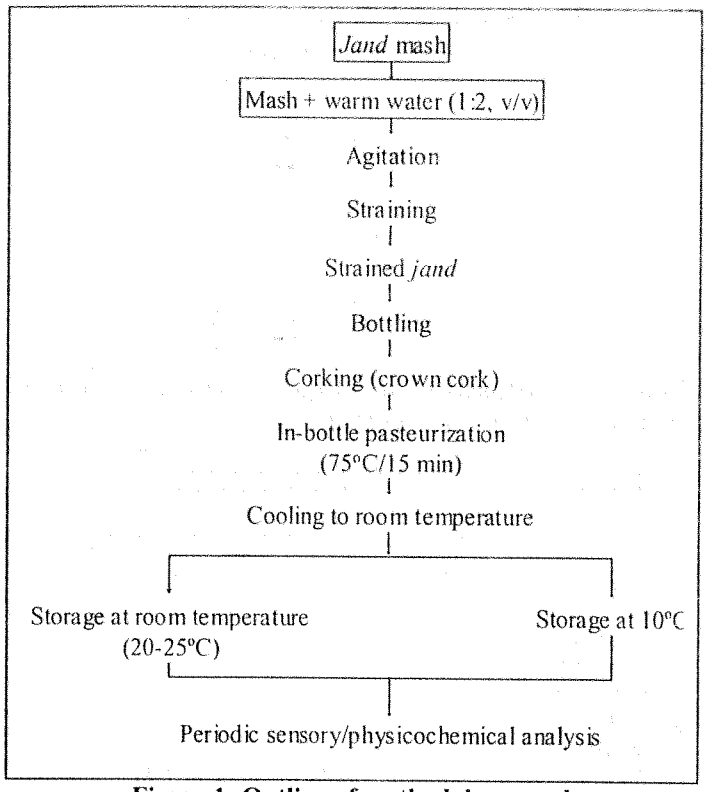

Figure 1: Outline of methodology used

Trial test: The mash was first extracted in tepid water (1:2, vol/vol). Physicochemical properties of the extract were noted and trial pasteurization carried out at different combinations of time and temperature using following variations:

1. Open-pot pasteurization
2. In-bottle pasteurization (bottles cotton-plugged)
3. In-bottle pasteurization (bottles crown-corked)

Used beer bottles were taken as the packaging material in the present study because these are the cheapest, the most robust (resistant to thermal shock), and the most readily available material in the rural areas of Nepal.

The physicochemical tests of the strained *jand* consisted of the determination of pH (direct readout meter), reducing sugars (Sathe, 1999), acidity (Pearson, 1981), alcohol content (Pearson, 1981; AOAC, 1980) and packed volume (Singh, 1998).

Selection of the best treatment was carried out using two criteria simultaneously, *viz.*, (i) retention of characteristics of strained *jand*, as ascertained by preliminary sensory test (color, flavor and homogeneity) by panels consisting of local connoisseurs, and (ii) lowest temperature needed to pasteurize the *jand*. The second criterion was verified indirectly by noting the absence of microbial activity (no carbon dioxide evolution when stored at 30°C for up to 3 days, after the pasteurization treatment). The lowest pasteurization heat treatment that showed absence of carbon dioxide evolution when held for 3 days at 30°C (Table 2) was selected for further experiments.

Final test: The samples of strained *jand* were prepared exactly as in the trial test and in-bottle pasteurized at 75°C for 15 min (the combination reached by trial, Table 2). The final test consisted of periodic comparison of the two sub-treatments of in-bottle pasteurized *jand* (stored at room temperature of 20-25°C and at 10°C) against control (freshly strained and pasteurized *jand*) in terms of organoleptic and physicochemical parameters. The testing was done every 12 days, up to 48 days. Figure 1 is an outline of the methodology used for the final test. Sensory evaluation was done for color, flavor and homogeneity on a 9 – point hedonic rating (9 = like extremely, 1 = dislike extremely) (Ranganna, 1999).

Statistical analysis: The ordinal values obtained for each of the tests were tested for difference by ANOVA and LSD (Gomez & Gomez, 1984; Gupta, 1994).

Results and Discussion

Trial study on preservation of strained *jand* revealed that open-pot pasteurization is unsuitable for preserving *jand* because of the loss in alcohol and *jand* characteristics. To retain *jand* of desired physicochemical characteristics given in Table 1, the best treatment was found to be in-bottle pasteurization at 75°C for 15 min (Table 2). Changes in physicochemical parameters of the treatments and control over a period of 48 days of storage (Tables 3, 4 and 5) showed no significant difference ($p < 0.05$).

Table 1: Physicochemical properties of strained *jand* used for the test

Parameters	[1]Value
pH	4.49 ± 0.03
Total acidity (m/vol.) as % lactic acid	0.61 ± 0.01
Reducing sugar (m/vol.) as % dextrose anhydrous	1.51 ± 0.02
Alcohol as % (vol./vol.)	4.04 ± 0.04
Packed volume as % (vol./vol.)	26.48 ± 0.34

[1]Value ± SD of three tests

Table 2: Time-temperature combination for in-bottle pasteurization of strained *jand*

Time-temperature combination (°C/min)	Gas bubbles on incubation (30°C for 3 days)			Sensory quality
	Day 1	Day 2	Day 3	
60/30	+	+	+	Normal
63/30	+	+	+	Normal
65/30	-	-	+	Normal
70/20	-	-	+	Normal
75/15	-	-	-	Normal
80/10	-	-	-	Normal

+ positive; - negative

Table 3: Physicochemical changes in in-bottle pasteurized *jand* during storage at ambient temperature (20-25°C)

Parameter	Storage period (days)				
	0	12	24	36	48
pH	4.50_a	4.51_a	4.51_a	4.51_a	4.50_a
Total acidity (m/vol.) as % lactic acid	0.60_a	0.60_a	0.60_a	0.61_a	0.61_a
Reducing sugar (m/vol.) as % dextrose anhydrous	1.52_a	1.51_a	1.50_a	1.49_a	1.50_a
Alcohol (% vol./vol.)	4.03_a	4.06_a	4.00_a	4.00_a	4.03_a

- Data are means of triplicates. Means followed by the same subscripts are not significantly different ($p < 0.05$)

The sensory quality of the treatments was significantly superior ($p > 0.05$) to control (Table 6) in terms of flavor but did not differ in terms of color and homogeneity. Treatments, irrespective of the storage temperature, did not differ significantly in terms of organoleptic quality.

Table 4: Physicochemical changes in in-bottle pasteurized *jand* during storage at low temperature (10°C)

Parameter	Storage period (days)				
	0	12	24	36	48
pH	4.50_a	4.50_a	4.51_a	4.52_a	4.51_a
Total acidity (m/vol.) as % lactic acid	0.60_a	0.61_a	0.61_a	0.60_a	0.62_a
Reducing sugar (m/vol.) as % dextrose anhydrous	1.50_a	1.52_a	1.51_a	1.50_a	1.51_a
Alcohol (% vol./vol.)	4.00_a	4.06_a	4.04_a	4.06_a	4.00_a

Table 5: Physicochemical changes in fermented mash of millet *jand* kept at – 4°C as control

Parameter	Storage period (days)				
	0	12	24	36	48
pH	4.44_a	4.45_a	4.45_a	4.43_a	4.43_a
Total acidity (m/vol.) as % lactic acid	0.60_a	0.61_a	0.61_a	0.62_a	0.61_a
Reducing sugar (m/vol.) as % dextrose anhydrous	1.48_a	1.47_a	1.49_a	1.48_a	1.43_a
Alcohol (% vol./vol.)	4.10_a	4.09_a	4.13_a	4.08_a	4.10_a

- Data are means of triplicates. Means followed by the same superscript are not significantly different ($p < 0.05$)
- Data are means of triplicates. Means followed by the same subscript are not significantly different ($p < 0.05$)

Table 6: Comparative overall mean scores of sensory evaluation of treatments and control

Day	Color			Flavor				Homogeneity		
	T1	T2	C	T1	T2	C	LSD (5%)	T1	T2	C
12	7.4_a	7.5_a	7.1_a	8.0_a	7.9_a	7.5_a		7.5_a	7.4_a	7.6_a
24	7.0_a	7.1_a	7.3_a	8.3_a	7.8_a	6.9	0.65	7.2_a	7.1_a	7.2_a
36	6.9_a	6.8_a	7.1_a	7.6_a	7.3_a	6.5	0.32	7.1_a	7.1_a	6.9_a
48	7.1_a	7.2_a	7.4_a	7.4_a	7.3_a	6.5	0.32	7.1_a	6.9_a	7.0_a

- The values are mean scores given by 12 semi-trained panelists
- Values followed by similar letters in the rows under each column (color/ flavor/ homogeneity) are not significantly different ($p < 0.05$)
- T1 = Treatment 1 (in-bottle pasteurized *jand* stored at 20-25°C)
- T2 = Treatment 2 (in-bottle pasteurized *jand* stored at 10°C)
- C = Control (freshly pasteurized *jand*, prepared from mash kept at – 4°C)
- LSD = Least Significant Difference

The overall results clearly indicate that strained *jand* can be preserved for a fairly long time even at room temperature if in-bottle pasteurization is done. The reason for this improvement could be largely attributed to the destruction or inactivation of microorganisms and their enzymes which cause the rapid deterioration of quality. A more detailed study is necessary to completely elucidate these effects. It is also possible that in-bottle pasteurization could have promoted 'forced maturation' as is the case with many wines.

Conclusions

In-bottle pasteurization appears to be a promising method for the preservation of strained *jand*. The method is very simple. An additional advantage of the method is that *jand* can be stored at room temperature, thereby eliminating the need for costly refrigeration. The finding indicates to the distinct possibility of preservation of *jand* by pasteurization. The time-temperature regime for pasteurization used in this study was reached by several trials conducted to retain desired physicochemical parameters. Admittedly, it is difficult to generalize a foolproof pasteurization scheme for *jand* of other physicochemical characteristics. In this light, the success-story of African beer can serve as an exemplary guideline for further R&D on *jand*.

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Preparation of Paneer from Sour Milk

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Paneer was prepared from sour milk with varying acidity levels of 0.25, 0.28, 0.32, 0.36, 0.40 and 0.44 % as lactic in this study. The study revealed that milk acidity affected the chemical parameters of paneer. Milk with initial acidity from 0.25 to 0.44 %, the yield of paneer decreased from 9.5 to 7.3 %. Similarly, moisture and fat were decreased from 55.0 to 49.0 % and 17.7 to 8.0 %, respectively. But an increase in the protein content from 22.2 to 29.5 % was observed. Ash ranged in between 2.7 to 3.1 %. Total plate count of 1×10^4 CFU/g was observed. Milk acidity had adverse effect on the texture, color, flavor, taste and overall quality of the product; primarily of taste and flavor. Sensorily, paneer made from milk with acidity below 0.32 % was acceptable.

Keywords: Paneer, Physicochemical composition, Yield, Sensory quality, Sourmilk Paneer

Introduction

Paneer is an important indigenous milk product, which is used as a basic material for the preparation of large number of culinary dishes (Sachdeva *et al.*, 1985). The process of paneer making consists of precipitation of milk with the help of suitable coagulant and separation of curd from whey. The curd is subsequently pressed for increasing the cohesion and removal of excess moisture (Kulshrestha *et al.*, 1987). Precipitation of milk at high temperature involves the formation of large structural aggregates of proteins in which milk fat; other colloidal and soluble solids are entrained with whey. Good quality paneer is characterized by a typical acidic flavor with slightly sweet taste, a firm cohesive and spongy body, and a closely-knit, smooth texture (Sachdeva & Singh, 1995).

As the milk is left undisturbed, clotting of the milk may occur due to developed acidity as a result of bacterial activity. If the milk is not processed it develops sour taste that becomes unacceptable to the consumers. One of the major challenges in the dairy industries in Nepal is the souring of milk that occurs mainly during transportation and storage. Due to this reason, there may be a great economy loss. On the other hand, the milk produced by the farmers cannot be totally utilized in the industries during peak production seasons. As a result, in a week for two days milk holiday has been announced by the milk processors. This excess of milk resulted from the milk holiday also develops acidity. Paneer preparation from this sour milk could be one of the greatest remedies. The objective of present study was to utilize sour milk by the preparation of paneer from varying level of acidity.

Methodology

Milk sample was collected from Kamdhenu Dairy, Tarahara. Citric acid was used as a coagulating agent.

Chemical analysis

Fat and solid-not-fat (SNF) of the milk received from Kamdhenu Dairy were determined by Gerber method (Egan *et al.*, 1981).

Acidity of milk sample was determined by the method given by Egan *et al.*, (1981). The pH of milk was determined by general method described elsewhere. Fat content in paneer was determined by Werner-Schmid method (Egan *et al.*, 1981). Acidity and pH of paneer was determined according to AOAC (1980). The protein in the paneer was determined by determining nitrogen content by Kjeldahl method and the multiplying by a factor 6.38 (Ranganna, 2000). The moisture and ash content of paneer were determined according to Egan *et al.*, (1981).

Sensory evaluation

The six different types of paneer samples prepared from the sour milk with titratable acidity of 0.25 %, 0.28 %, 0.32 %, 0.36 %, 0.40 % and 0.44 % as lactic acid were named as A, B, C, D, E, F, respectively. These were organoleptically evaluated for the consumer's acceptability of product by using hedonic rating scale (Ranganna, 2000). For this, seven semi-trained panelists were first trained about product quality and asked to evaluate the product according to the evaluation card (Ranganna, 2000).

Microbiological analysis

Total Plate Count of the paneer samples was observed according to Eckles *et al.*, (2000).

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Statistical analysis

Statistical analysis of all parameters analyzed was carried out using one-way ANOVA at the 5 % level of significance according to Ranganna (2000).

Preparation of sour milk paneer

Milk sample was left overnight at room temperature for souring. The titratable acidity was determined in the following day at the end of souring period with acidity 0.25 % for preparing first sample of paneer. The milk was further allowed to stand till the acidity reached 0.28 % for preparing second sample. Similarly, the milk was allowed to stand further till the acidity reached 0.32, 0.36, 0.40 and 0.44 % for the next four samples respectively. Each soured milk was heated separately to 71°C and coagulated by adding 40 ml/kg warm (60 - 65°C) citric acid (1 %) solution with continuous stirring. It was then left for 5 minutes to separate clear whey and coagulum. The coagulum so obtained was filtered through muslin cloth. It was then kept in hot water (65°C) for 5 minutes for washing. It was then pressed in the wooden box (hoop) by weight about 2 kg/cm^2 for 25 minutes. The obtained mass was then cut into small cubic pieces of 8 cm^3. The pieces were immersed into chilled NaCl solution (5 %) at 4 – 6°C for 2 hours. The paneer pieces were taken out and drained. Surface excess moisture was removed by cleaned muslin cloth before packaging with polyethylene bags and kept in refrigeration.

Results and discussion

Relation between time of holding with pH and acidity

The relation between time of holding with pH and acidity is shown in Figure 1. When the milk was allowed to stand, there was a gradual increase in acidity as well as gradual decrease in pH. The increase in acidity or decrease in pH was due to the fermentation of milk sugar (lactose) to lactic acid by the bacteria present in the milk.

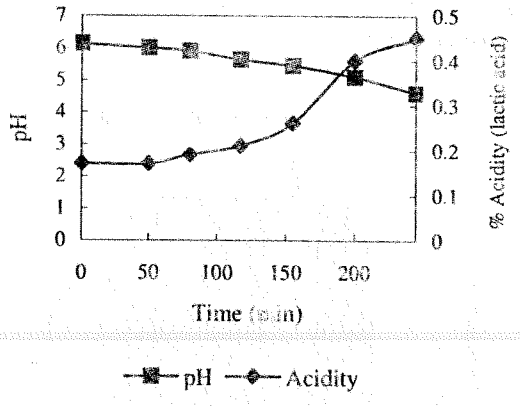


Figure 1: Relation between time of holding with pH and acidity

Effect of acidity on the total plate count of paneer

The effect of acidity on the log colony forming units (CFU) per gram of the product is shown in Figure 2. Initially there was a decrease in the microbial population and then an increase followed by a decrease again. But overall, there was decrease in the total population with the increase in acidity of milk.

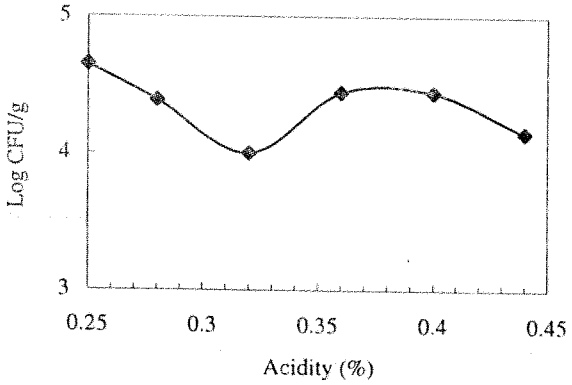


Figure 2: Effect of milk acidity on the total plate count (TPC) of paneer

Moreover, the overall population suffered a decrease from lower acidity levels to higher ones. It was due to the continuous inhibition due to addition of lactic acid. Frazier (1995) reported that, at room temperature, an acid fermentation in milk is most probable, first by lactic Streptococci and Coliform bacteria and then by the acid tolerant Lactobacilli. Then molds or film yeasts on the surface lower the acidity permitting the formation of more acid. Eventually, when most of the acid has been destroyed, proteolytic or putrefactive bacteria complete the decomposition.

The effect of milk acidity on the chemical composition and yield of paneer

Six types of paneer prepared from the milk containing 0.25, 0.28, 0.32, 0.36, 0.40 and 0.44 % acidity were coded as A, B, C, D, E and F, respectively. They were then analyzed for their chemical composition and the yield.

The effect acidity on the moisture content of paneer

The effect of milk acidity on the moisture content of paneer is shown in Figure 3. It has been found that with increasing acidity, moisture retention was decreased. The retention of the moisture in paneer was due to the moisture present in the structure of protein in contact with curd particles and free moisture present in paneer (Scott, 1985).

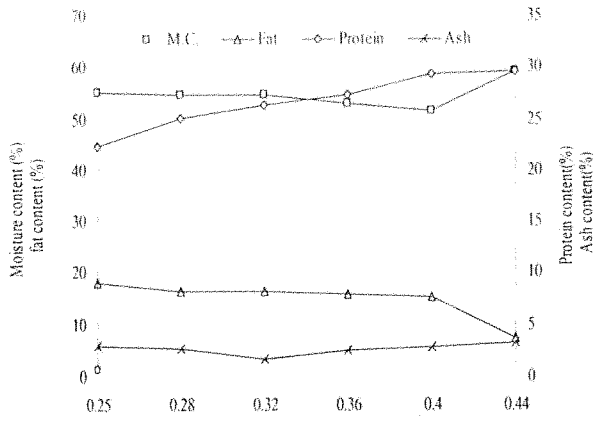


Figure 3: Effect of acidity levels on chemical composition of paneer

The effect of acidity on the fat content of paneer

The fat content of the paneer declined gradually with the increase in the level of milk acidity (Figure 3).The decrease in fat percentage might be due to the degradation of fat into different compounds. Milk has complex physicochemical properties and during fermentation, the fat also degrades to different aroma producing substances due to lactic acid and other bacteria. Also, the acidity itself may have the effect in holding the fat in the product (De, 2000). This implies that, there is more tendency of separation of fat and hence, more fat loss in the paneer.

The effect of acidity on the protein content of paneer

The percentage of protein was found to be increased steadily with the increase in the acidity of milk (Figure 3). While preparing paneer, it was found that, on heating the milk with acidity ranging from 0.25 % to 0.44 %, there was formation of denser and thicker curd from the milk containing higher level of acidity. This caused more moisture expulsion from the curd. With lower acidity, there was no proper coagulation during heating. Nielson and Ullum (1989) reported that with a decrease in pH the casein precipitates at lower temperatures while heating. Also, there was decrease in fat content with increasing acidity. Due to these, the paneer prepared from the milk with higher acidity resulted a higher percentage of protein.

The effect of acidity on the total ash content of paneer

The total ash content in different samples of paneer made from milk with different levels of acidity is shown in Figure 3. It was found that there was a slight decrease followed by an increase in the ash content of the paneer. De (2000) reported that the ash content of product depends upon the mineral content in the raw material that may vary from species to species, breed, feed etc. Since these conditions were constant, the variation in the ash content may be due to the improper balance in the total salt present in the milk and that taken up during brining.

The effect of milk acidity on yield of paneer

The effect of different milk acidities to the yield of paneer is shown on Figure 4. It was found that there was a gradual decrease in the percentage yield from 9.5 to 7.3 on the increase in the percentage of titratable acidity of milk from 0.25 to 0.44 %. When there was increase is the acidity or decrease on pH, the total solid recovery was decreased. As a consequence, there was decrease in the yield. Ghodekar (1989) reported that the yield mainly depends upon the type of milk used, heat treatments, coagulant type and its concentration etc., these are known to vary to a great extent. In this case, all the other factors except the amount of coagulant (acidity) were constant; hence increase is acidity was the basic cause of decrease in the yield. Similar results were reported by Mohd *et al.*, (1985) and Sachdeva and Singh (1987).

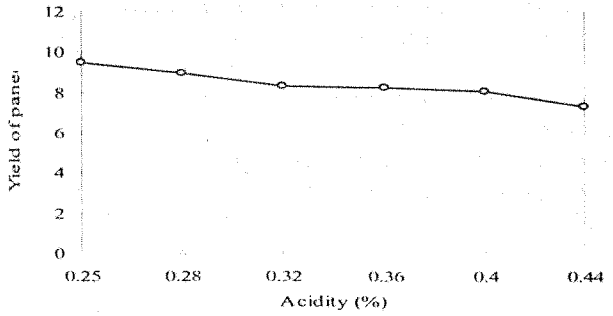
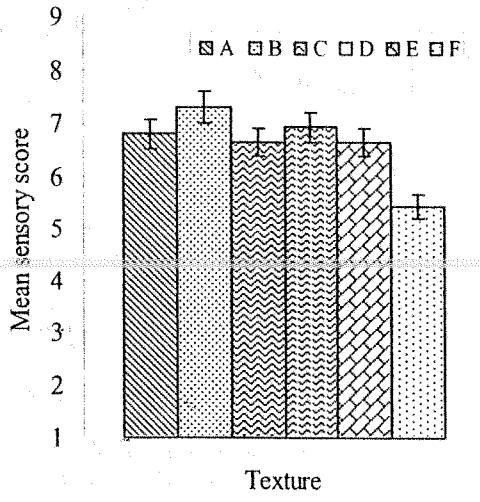


Figure 4: Effect of increasing acidity on the yield of paneer

Sensory Evaluation

The samples of paneer prepared from milk having titratable acidity of 0.25, 0.28, 0.32, 0.36, 0.40 and 0.44 % were coded as A, B, C, D, E and F, respectively. These were then asked for seven semi-trained panelists. They were asked to score for the sensory parameters such as texture, color, flavor, taste and overall quality of the sample. The analyses of sensory parameters were carried out so as to select the product that is prepared from the highest level of milk acidity and significantly indifferent with the highest scoring product. The result of sensory evaluation is presented in Figure 5. Texture of all samples A, B, C, D and E were not significantly different ($P<0.05$) except F. The average mean score was greatest for sample B.



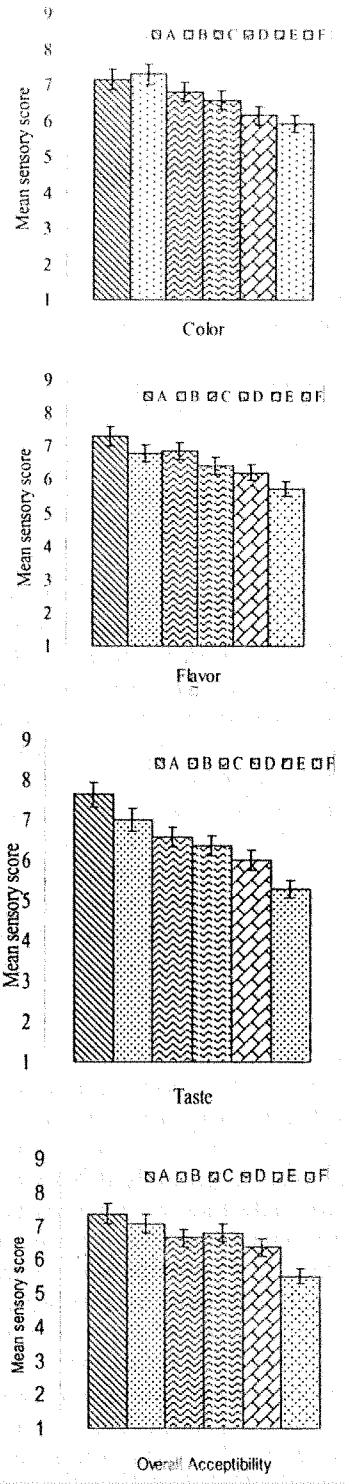


Figure 5: Effect of milk acidity on mean sensory score of paneer

In case of color, the products, A, B, C and D were not significantly different each other. But the other two products E and F were significantly different ($p < 0.05$). The color was not much affected at initial acidity levels but was unappealing at higher levels. The average mean score was greatest for sample A.

The samples A, B and C were not significantly differ in respect of flavor and taste, but the samples D, E and F were significantly($p < 0.05$) differed. In overall quality products A, B, C, and D were not significantly ($p < 0.05$) differ whereas, E and F was differ. There was very slight difference between A and B. Except in case of the texture, sample A had the highest mean score. It showed that the consumers' acceptability is greatest towards the paneer made from less acidic milk. Since taste and flavor can be taken as more important parameters for the selection of paneer. The selection of the product was made on the basis of the sensory scores. Moreover, with the

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Monitoring Good Manufacturing Practices of Dairy Industries in Nepal [#]

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The study highlights the situation of good manufacturing practices of dairy industries in Nepal which showed an immediate need for proper documentation of all the products and also need to follow control measures performed during processing. It was found that pasteurized milk product contains high number of coliform bacteria, which also indicated possibility of improper pasteurization and severe post contamination problem. Best practice to prevent such problem is Quality Assurance Programme, which embodies of integrated GMP-HACCP module in the simplified form of code of practice in a phased manner.

Key words: GMP, Coliform, Code of Practice, GMP-HACCP, Dairy Industires, Nepal

Introduction

The history of dairy development is not so long in Nepal. First dairy was started in Tushal (Kavre). In 1956, first milk processing plant was established at Lainchaur, Kathmandu with a capacity of 500 litres. In Nepal, there are above 200 dairies industries with the capacity as much as 100 litres or more per day. They categorize industries according to the capital investment. All fall in the same categories even though they posses different physical facilities and their production is 50 to 100 litres per day or 30 to 100 thousands litre per year (Shakya & Shrestha, 2003).

About 1,170,000 MT of milk is produced in Nepal annually. Only about 10% production enters into processing. About 300,000 litres of milk are consumed daily in Kathmandu valley whereas; Dairy Development Corporation (DDC) and private sector dairies produce 150,000 litres each. Approximately 50,000 litres milk is supplied directly by the small venders and farmer themselves (Shakya & Shrestha, 2003). Dairy sector has contributed 6% of the national GDP (NDDB, 2001). Dairy sector is one of the important sectors, which has helped to reduce poverty and helped to increase the living standard of the rural people.

The mandatory standard of pasteurized milk has been fixed at 2029 B.S. Since then it has been routinely monitoring by Department of Food Technology and Quality Control (DFTQC) as a part of its activities to prevent from adulteration and to ensure supply of wholesome product for consumers. The adherence to total solid, fat and pasteurization test has become like a stumbling block to maintain it as per the food standard besides complying the requirement of labeling.

There is no other better option rather than implementing code of practice as early as possible. Even the food regulation under review takes into account implementing elements of preventive dimension of quality management and inspection such as code of practice under mandatory surveillance mechanism. Thus code of practice may take the form of establishing inspectional evidence if such violations are repeated. The quality management system has embraced the use and application of GMP and HACCP system as a viable tool to upgrade quality and safety of the product. In this study, an attempt has been made to monitor dairy industries with the objective to study good manufacturing practices of pasteurized milk throught the milk chain including different stages of processing.

Methodology

The study was carried out by both the primary and secondary data information. The primary data information was collected by the GMP team (Senior Food Research Officer, Food Research Officer, Food Inspector and Microbiologist) for the inspection of dairy industries of Kathmandu valley with a questionnaire and an inspection form that were approved by the technical committee. The inspected dairy industries included both large and small. The milk samples were collected from different critical control points of dairy industries. The samples were stored in cool box as soon as they were collected and analyzed in Central Food Laboratory of the Department of Food Technology and Quality Control, Babar Mahal, Kathmandu. The analyzed parameters of samples were as follows:

a. *Chemical*: - Milk Fat
- Milk Solid Not Fat

b. *Microbiological*: - Coliform (Count/ml.)

The chemical and microbiological analysis was carried out by the method referred to FLM, 2004; IDF, 1996; AOAC, 1984.

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[#] The work reported in this paper is a part of the work carried out for Implementation of Code of Practice for Assuring In-Process Quality Management of Dairy Products, Organized by Department of Food Technology and Quality control Babarmahal, Kathmandu, Nepal in Co-operation with WHO.

Results and Discussion

Participating dairies in the monitoring scheme were provided orientation on Good Manufacturing Practice (GMP) and Good Hygienic Practice (GHP) at the collection of raw milk, and subsequent processing (pasteurization, filling, storage, and distribution) etc. The general principle of CIP (clean-in-place), and cleaning of pipelines at the processing plants, were given due importance in improving hygienic condition and safety of the pasteurized milk. During the monitoring of milk quality, samples were collected from the various stages of milk processing & from the market and analyzed in the Central Food Laboratory, DFTQC. The monitoring of hygienic condition at various stages of processing (raw milk, after pasteurization, before and after filling at pouches) indicated a very interesting result in the major dairies of Nepal. The most probable number (MPN) of coliform at the raw milk was almost more than 2400 in all the dairies indicating the similarities of hygienic condition at the farm level (Table 1 and 2). The monitoring of coliform was carried out at three different time period (Quarterly). In the first monitoring programme, the coliform count was high in majority of the dairies except one. The result was reviewed and provided additional technical guidance for further improvement of hygienic condition of the milk. In the second monitoring programme, there was moderate improvement in dairies excepting in before and after filling stage. But at the third monitoring programme, the result was satisfactory in all the leading dairies of Kathmandu (Figure 1).

Table 1: GMP monitoring of big dairies in Nepal

Processing stage	Coliform present/Total Sample				Coliform count/ml			
	A	*B*	*C*	*D*	*A*	*B*	*C*	*D*
Raw Milk	3/3	3/3	3/3	3/3	>2400	>2400	>2400	>2400
After Pasteurization	2/3	2/3	1/3	1/3	0 - 100	0 - 2400	0 – 2400	0 – 2400
Before Filling	3/3	2/3	3/3	1/3	50 - 100	0 - 2400	200 – 2400	0 – 2400
After Filling	3/3	2/3	3/3	3/3	100 - 400	0 – 2400	500 – 2400	50 – 2400
Market Distribution	7/28	2/2	14/40	6/6	4 - 1100	9 - 2400	9 - 2400	4 - 2400
Total	**18/40**	**11/14**	**24/52**	**14/18**				

Table 2: GMP monitoring of small dairies in Nepal

Processing stage	Coliform present/Total Sample				Coliform count/ml			
	E	*F*	*G*	*H*	*E*	*F*	*G*	*H*
Raw Milk	1/1	1/1	1/1	1/1	>2400	>2400	>2400	>2400
After Pasteurization	0/1	0/1	0/1	0/1	0	0	0	0
Before Filling	1/1	0/1	1/1	0/1	28	0	43	0
After Filling	1/1	1/1	1/1	1/1	2400	460	2400	100
Market Distribution	2/2	12/12	2/2	2/2	50 - 2400	50 - 2400	100 - 2400	50 - 1100
Total	**5/6**	**14/16**	**5/6**	**4/6**				

In case of monitoring of hygienic condition of four small dairies at various stages revealed the result indicating similar to that of leading bigger scale dairy processing plants in terms of maintaining hygienic condition and sanitation of the plant at various stages of processing (raw milk, pasteurization, and filling etc.). The performance of the small dairies was quite satisfactory; and those dairies required more attention to improve the hygiene and safety of the finished pasteurized milk (Figure 2).

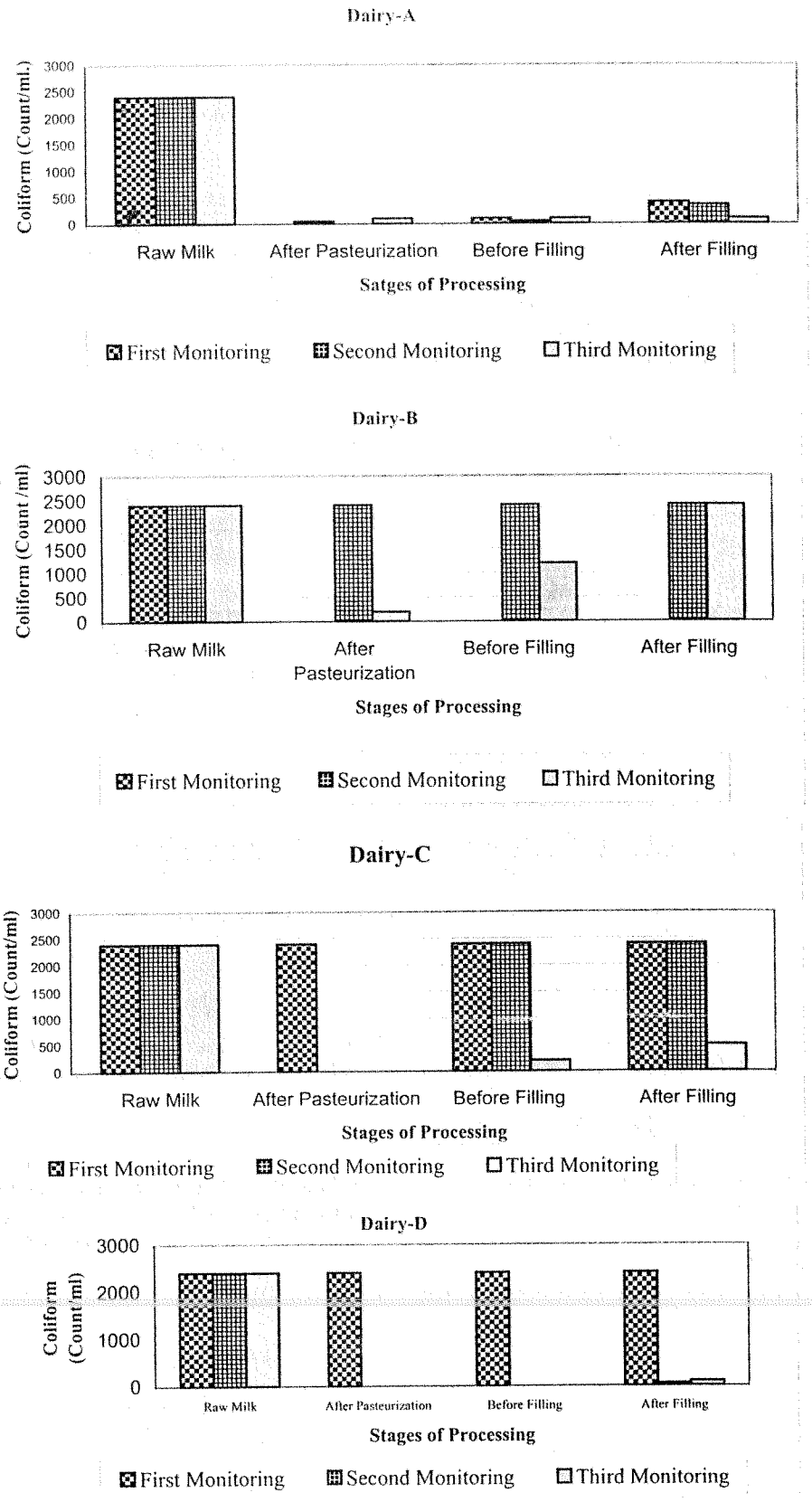

Figure 1: GMP monitoring of major dairies during the various stages of processing

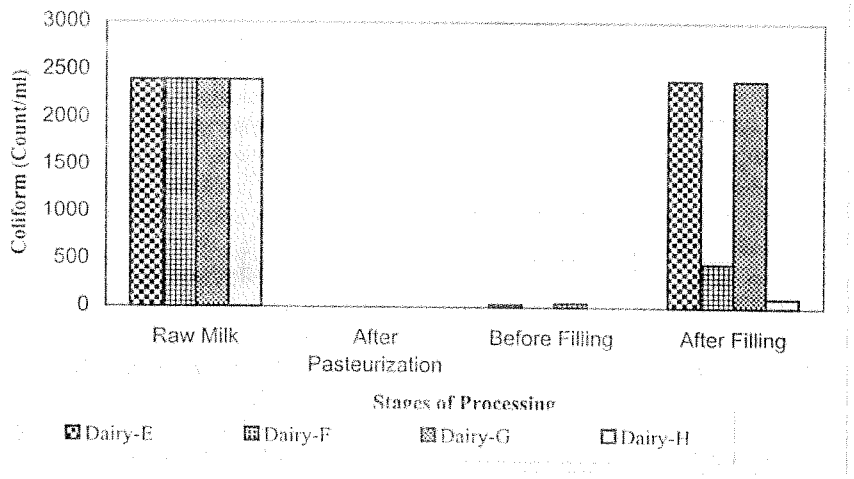

Figure 2: GMP monitoring of small dairies during the various stages of processing

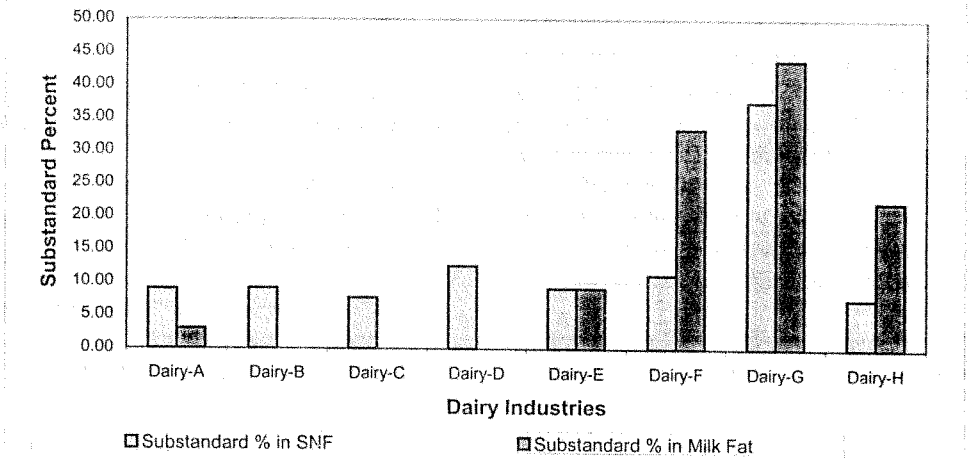

Figure 3: Comparison of SNF and milk fat in pasteurized milk of dairy industries

Physical structures of big dairies were quite satisfactory from the point of view of their construction. Environmental conditions of these dairies were satisfactory. Most of big dairies had water treatment system; the tanker in controlled temperature collected raw milk and also had skilled employees and competent personnel but effuluent treatment system was acking. Pasteurization temperature used was found to be more than 72°C. Pasteurized milk was stored in controlled temperature tanker.

Milk was stored few hours before filling. Packed milk was held in refrigerated room before distribution. However, cool chain was not maintained during distribution. Most of dairies had microbiology laboratory and routine tests were carried out. CIP system was used for cleaning pipelines and equipments. Using of aprons and appropriate clothing were found in these dairies. The major milk products produced by these dairies were milk, butter, ghee and curd although they were concentrated on fluid milk with insignificant production of milk products.

However, physical structure of small-scale dairies were not satisfactory in terms of construction, processing rooms were very congested. Water treatment system was lacking. Temperature controlled tankers were not used during raw milk collection. Skilled manpower and microbiology laboratory was lacking and also cool chain was not maintained during dispatch-processed milk. All the other dairies had continuous process excepting one Dairy where there was lack of that facility for milk storage during processing and before distribution. Most of the small-scale dairies were composite types and manufacture variety of milk products but mostly concentrated in fluid milk as big dairies.

The pasteurized milk samples after filling were collected from four large scale dairies and four small scale dairies and analyzed the SNF and Milk Fat content comparing with HMG standard which are not less than 8 and 3 percent respectively. Around 9 % samples were substandard in term of SNF content. In term of

Milk Fat , all samples collected from three dairies were within standard; however around 33% samples from small dairies where they did not have their own proper lab facilities were substandard in terms of milk fat.

Observing all these dairies we have found that most of the dairies have focused only on fat and SNF content, there is no strict and systematic monitoring for bacterial quality though some dairy posses microbiological laboratory (Figure 3). Small-scale dairies do not have quality control laboratory at the plant and not aware of analyzing milk products in private laboratories. There is also a need for proper documentation of all the products that are practiced in the plants and control measures performed during processing. It was seen that pasteurized milk product contains high number of coliform bacteria, which also indicated possibility off pasteurization and sever post contamination problem. Overall, best practice to prevent such problem is Quality Assurance Programme, which embodies of integrated GMP-HACCP module in the simplified form of code of practice in a phased manner.

Conclusions

Improvement of hygiene and safety of processed milk require an integrated approach and monitoring scheme. The integrated measure of GMP and HACCP need to be practiced in the processing plants promptly and effectively. Unless this practice is translated into daily action, the task of improving safety and hygienic condition will remain a far-fetched destination. Thus GMP-HACCP module is the must, and should be practiced by all processors, manufactures and handlers.

The selling/ distribution of milk and milk products should be encouraged through cold store, super market, and departmental store. Constant vigilance in term of chemical and microbiological examination of raw materials, intermediate products and final products should be strictly pursued. All pasteurized milk product should be stored and marketed in an unbroken cooling chain after heat treatment in order to keep bacterial growth as low as possible and ensure shelf life of the milk products as long as possible. Adaptation of code of practice should be made obligatory to all dairies to ensure wholesome milk production.

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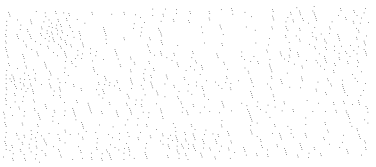

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