

**STUDIES ON THE MICROFLORA OF SOME
TRADITIONAL FERMENTED FOODS OF
DARJEELING HILLS AND SIKKIM**

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Certified that the work presented in the thesis, entitled 'Studies on the microflora of some traditional fermented foods of Darjeeling hills and Sikkim' has been carried out by Mr Jyoti Prakash Tamang, M.Sc. under my supervision at the Department of Botany, University of North Bengal. The results incorporated in the dissertation have not been submitted for any other degree elsewhere.

Further certified that Mr Tamang has followed the rules and regulations laid down by the University of North Bengal in carrying out the work.



Dr P.K. Sarkar

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1. INTRODUCTION

Fermented foods provide a major contribution to the diet in all parts of the world. A number of food fermentation processes including those that yield cheese, yoghurt, sausages, pickles, sauerkraut, soya sauce, bread and beer have been extensively investigated and accurately documented. But, many other foods prepared by the action of bacteria, yeasts and moulds on plant materials have been neglected, mainly because they are almost totally unknown outside their native countries (Hesseltine and Wang 1980).

A food is considered fermented when one or more of its constituents have been acted upon by microorganisms to produce a considerably altered final product acceptable for human use (van Veen 1957). Traditional fermentation processes are those which have been used for centuries, even before it was recorded in history (Hesseltine and Wang 1980). Most of these processes were developed long before the existence of microorganisms was recognized (Wang and Hesseltine 1981).

Ever since man started hunting and gathering food to organized food cultivation, preservation in the forms of drying, salting and fermentation was developed (Campbell-Platt 1987). Descriptions of fermented foods go back as far in time as inscriptions are available. The Egyptians, Sumarians, Babylonians and Assyrians knew about the use of barley to produce alcoholic beverages; a cuneiform inscription on a Babylonian brick from 2800 B.C. gave a recipe for the production of barley wine (Borgstrom 1968).

Records of soya sauce and also miso production in China go back to around 1000 B.C. with the transfer of knowledge of these production processes to Japan occurring around 600 A.D. (Yokotsuka 1985).

Early Europeans were known to be making flat sour-dough bread from rye in 800 B.C. Around 100 B.C., there were 250 bread bakeries operating in Ancient Rome (Pederson 1979). Accounts of using dahi (curd) throughout India, dadhanvat (milk product) and kali (fermented rice) in southern part of India and panir (milk product) in Punjab are as old as 2000 B.C. (Om Prakash 1961). Records of dosa and idli go back to 1100 A.D. (Gode 1955). The history of using bhat bajraka (bajra flour product) in southern India, rabdi (maize flour product) in Rajasthan and rasgulla (milk product) in Bengal is found in 1500 A.D. (Pathak 1970). As soon as man started collecting milk from animals, sour milk became an item in his diet. Accounts of production of fermented dairy products can be found in early Sanskrit and Christian works, while recipes of soured fermented milks were given in Roman times around 200 A.D. (Oberman 1985).

The essential objectives of developing traditional fermentation technology were to carry over supplies from the time of plenty to those of want. It transpires an essence of knowledge and wisdom, gained by experience and based on trial and error. People might not be able to explain what is going on during storage and processing in terms of scientific language, but they certainly know what they have to do to get the desired product (Dietz 1984). The fermentation techniques pass as a trade from older to younger generations in the families; and in some regions of India, the hereditary nature of the profession preparing fermented foods is not only encouraged

but also protected by tradition or secular means (Batra and Millner 1976). These methods were based on interdependent factors, such as available raw materials, climate, available energy source, topography, culture and religion.

Traditional fermented foods form the basic components of the diet, and provide major nutrients to the people of many countries where hunger and malnutrition accompany poverty. With increase in population and paucity of protein sources, inexpensive and easy-made traditional fermented foods are becoming popular even in the developed countries.

The growth and activity of microorganisms play an essential role in controlling the whole environment and ecosystem, of which food supply is only a part. Microorganisms are responsible for the biochemical changes which occur during fermentation (Campbell-Platt 1987). Typically, the microorganisms used are those present in or on the ingredients and are selected by adjusting the fermentation condition (Hesseltine and Wang 1980). Microorganisms bring about specific transformations of the substrates for which traditional fermented foods are prized for the following advantages:

1. Microorganisms produce desirable enzymes: during miso production, both protein of soya bean and starch of rice are hydrolysed by the enzymes produced by *Aspergillus oryzae* (Hesseltine and Wang 1967).
2. Microorganisms destroy undesirable components: during tempe preparation, trypsin inhibitor is inactivated by *Rhizopus oryzae*, and factors causing flatulence are eliminated (Hesseltine 1983a).
3. Fermented foods provide dietary variety where choice of food is limited (Hesseltine 1965). Masking of any undesirable flavour or

production of any improved flavour of the fermented foods helps to overcome the monotony of eating local plant products (Hesseltine 1979). In many fermentations using soya beans, the undesirable beany flavour is replaced by a pleasant, nutty flavour (Hesseltine 1983a).

4. Many of the fermented foods are preserved without refrigeration or other energy-intensive operations because of reduction of pH and production of organic acids by lactic acid bacteria (Hesseltine 1979). Presence of antimicrobial agents, often produced by the fermenting microorganisms, prevent the growth of toxin-producing bacteria (Hesseltine 1983a).

5. Fermentation enhances nutritional value. Many microorganisms synthesize vitamins: in tempe, the levels of niacin, riboflavin and cyanocobalamine are increased (van Veen and Steinkraus 1970). In soya idli, in addition to these vitamins, an increased level of amino nitrogen and free sugar was found (Ramakrishnan 1979).

6. Fermentation increases digestibility of the product: during tempe and natto production, proteins are broken down to amino acids (Hayashi 1977; Steinkraus 1983a).

7. Fermentation may change the physical state of a product: in tempe, loose soya bean flakes or cotyledons are bound together to make a solid cake which when cut resemble non-textured bacon slices. In making soya sauce, components of solid substrates are digested by the koji enzymes into more soluble compounds resulting in a tasty liquid product (Hesseltine 1983a).

8. Fermentation produces colour to the product: in making angkak, dehulled rice is fermented with *Monascus purpureus* which imparts a brilliant purple-red water-soluble colour (Beuchat 1978); this

product is used in colouring meats and rice wine (Hesseltine 1983b).

9. Fermentation may produce alcohol: a number of rice fermentations including tape of Indonesia and lao-chao of China are carried out by amylolytic filamentous fungi in combination with alcohol-producing yeasts (Wang and Hesseltine 1981).

10. Some of the traditional fermented foods require less energy than conventional processes. Generally, the lactic acid fermentations are low-cost processes, and often little or no heat is required in them. Thus, they are fuel-efficient (Steinkraus 1983b).

11. Some traditional fermented foods have medicinal uses. In Russia, koumiss, a fizzy alcoholic beverage made from horse's, donkey's or camel's milk, has been used widely in treating pulmonary tuberculosis (Kosikowski 1977). Kvass, an alcoholic beverage of Russia made from rye or wheat bread, affords protection to the digestive tract against cancer (Wood and Hodge 1985).

12. Some of the traditional fermented foods are sun dried. Then, they can be transported easily from one place to another (Hesseltine 1979).

13. Traditional fermented foods have been prepared since prehistoric time and, therefore, are culturally acceptable. Most Indian Hindus eat only non-animal foods. Hence, products made from cereals and legumes are acceptable (Hesseltine 1979).

Darjeeling, with an area of 3075 km² and a population of 1,335,618 (Census of India 1991b) is a district of West Bengal in India. Excepting Siliguri, the three other subdivisions of this district including Darjeeling, Kalimpong and Kurseong are hilly (commonly, Darjeeling hills), inhabiting 70% of the district population. Sikkim, a tiny Himalayan state of india, with an area of 7096 km² and a population of 403,612 (Census of India 1991a)

lies north to Darjeeling hills (Fig. 1). It comprises four districts including East, West, North and South. Topographically, culturally and ethnically, people of Darjeeling hills and Sikkim, comprising of mostly the Nepalis, the Bhutias and the Lepchas, have remarkable similarities.

The food habit of a region is, to some extent, reflected in the pattern of food production. The staple crop in these regions is maize (*Zea mays* L.) followed by paddy (*Oryza sativa* L.) which is cultivated mostly in the lower altitudes of both Darjeeling hills and Sikkim. Depending on the altitude, wheat (*Triticum aestivum* L.), barley (*Hordeum vulgare* L.) and finger millet (*Eleusine coracana* Gaertn.) are cultivated. A variety of legumes including soya bean [*Glycine max* (L.) Merrill] and black gram [*Vigna mungo* (L.) Hepper] are also grown. The main vegetables cultivated during autumn and winter are 'rayo' [*Brassica rapa* L. ssp. *campestris* (L.) Clapham var. *cuneifolia* Roxb.], mustard [*Brassica rapa* L. ssp. *campestris* (L.) Clapham] and radish (*Raphanus sativus* L.). In the low-altitude areas, different varieties of bamboo are grown; some of them, when young in monsoon, are edible. Table 1 shows the area and annual production of some of the crops commonly used as substrates for preparation of traditional fermented foods of these regions.

Besides agriculture farming, cattle rearing is common for milk, milk products and meat. Goat, pig and sheep rearing, usually for meat, is also common. In the northern parts of Sikkim, bordering arid Tibetan plateau and in the north-eastern parts of Darjeeling hills, at an altitude of >4000 m, yak rearing is common for the

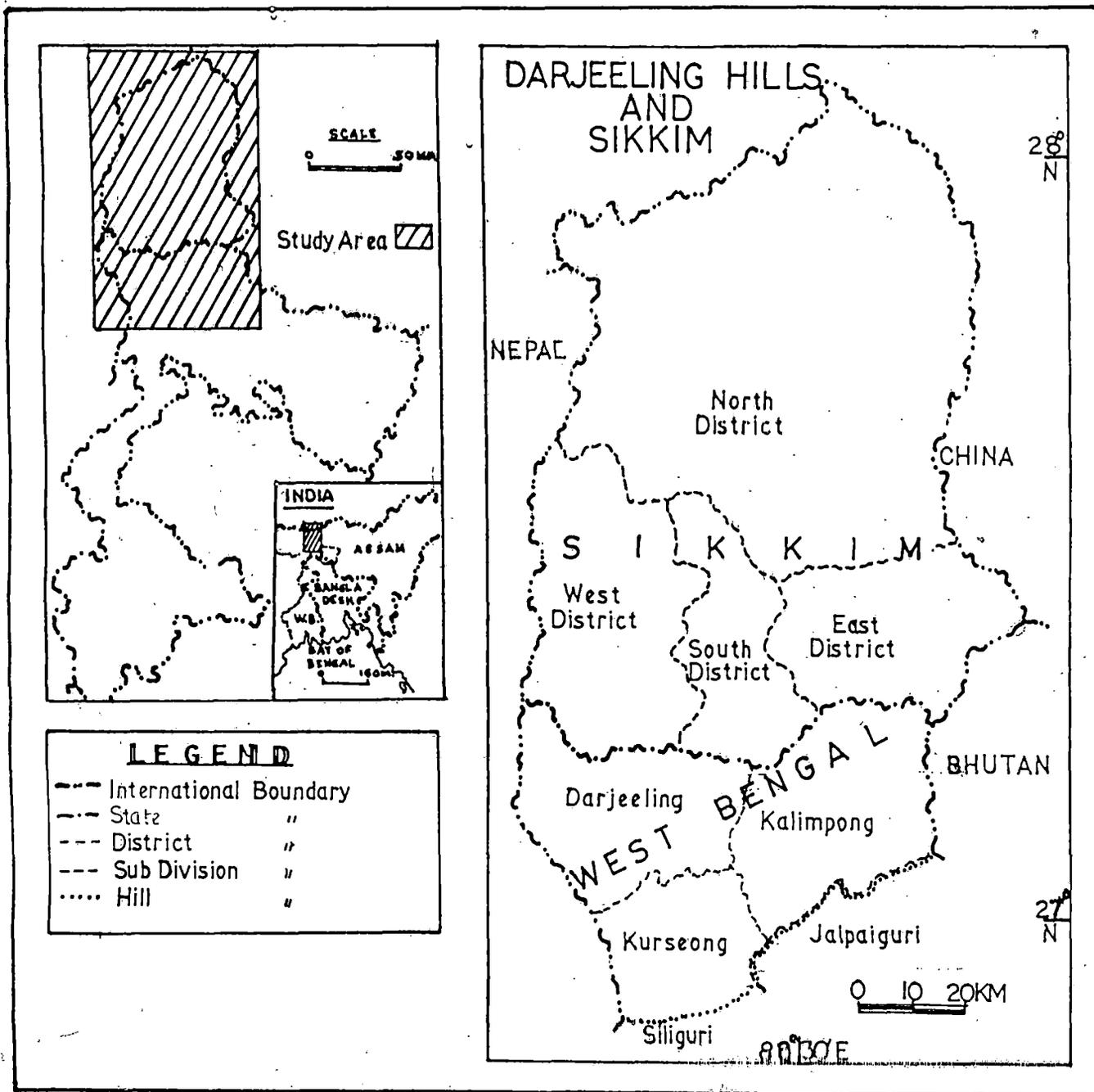


Fig. 1. The location map of Darjeeling hills and Sikkim

Table 1. Area and production of some of the agricultural produces of Darjeeling hills and Sikkim in the year 1990-91, used for the preparation of traditional fermented foods

Agricultural produces	Darjeeling hills ^a			Sikkim ^b		
	Area (ha)	Production (t)	Cultivar	Area (ha)	Production (t)	Cultivar
Rice	9,107	15,637	Local: Adday Ramtulsi Jhapaka Improved: C1 5310 Kalimpong 1	18,610	25,300	Local: Adday Dormali Dutkali Improved: Giza 14 Pusa 33
Soya beans	392	255	Local: yellow brown	4,680	7,600	Local: yellow brown Improved: Bragg Gyalab
Leafy vegetables	243	1,944	Local	400	3,000	Local
Radish	398	1,463	Local: Tibetan mula muli Japanese white	300	1,800	Local: Tibetan mula muli Japanese white

^aSource: Office of the Principal Agricultural Officer, Government of West Bengal, Darjeeling

^bSource: Department of Agriculture, Government of Sikkim, Krishi-Bhawan, Gangtok

same purpose. However, the use of meat is a taboo to some of the Hindus inhabiting these places.

Fermentation of vegetables, cereals, legumes, dairy products and meat has been practised in these regions since a long time. These fermented foods provide basic diet as staples, adjuncts to staples, condiments and beverages for the people of these regions. Some ethnic groups are economically dependent upon these fermented products. Despite rapid urbanization, traditional fermented foods still have ethnic importance and are essential items in their diet. Solemnizing marriages, and even worshipping the Gods and Goddesses by offering fermented beverages are traditional practices still common among some ethnic groups of these places.

The traditional fermented foods of these regions include kinema (soya bean product), masayura (black gram product), gundruk (leafy vegetable product), sinki (radish tap root product), mesu (bamboo shoot product), khalpi (cucumber product), shel roti (rice preparation), dahi, kachcha churpi, dudh churpi, churpi (all dairy products), sukako masu (meat product), murcha (starter culture), jnard (finger millet product) and raksi (rice beer).

The production of these foods has remained a traditional family art practised in homes in a crude manner. Consequently, the production has not increased substantially, the quality is not consistent and the shelf-life is short. In order to scale-up their production, it is necessary to modernize the production style and optimize the process conditions. In order to know how the preparation could be modernized, it is necessary to know the scientific basis of fermentation. This will shorten production time, guarantee

improved and consistent flavour and nutritional value, increased shelf-life, and this in turn will increase their general acceptability.

Most of these traditional fermented foods were not even so far documented. The present investigation is an attempt to bring some light into these obscure yet important foods. It aims to study the traditional methods of their preparation, modes of consumption, ethnic values, and microbial and selected biochemical associations in some of them. The information obtained will provide relevant background data for the development of improved fermentation technology.

Strategies proposed to be adopted for accomplishing the above objectives are:

1. Obtaining information in as much details as possible on the methods used by the local people to prepare the foods, modes of consumption and ethnic value of various traditional fermented foods;
2. Studying proximate composition and isolating dominant microorganism(s) of some foods;
3. Optimizing traditional process parameters;
4. Studying succession of microflora and selected biochemical parameters;
5. Testing each of the isolates or combinations thereof by producing the native product in the traditional way;
6. Characterizing the proven producing strain(s) in order to identify their taxonomic status; and
7. Improving the foods by using selected pure culture strain(s) to replace the mixture with undesirable ones now in use.

2. REVIEW OF LITERATURE

Many of the traditional fermented foods of Darjeeling hills and Sikkim have similarities with several fermented foods of the Orient, Africa and Europe (Table 2). Since most of the foods under investigation had no written record, mainly the literatures of their similar products were reviewed.

2.1. FERMENTED LEGUMES

2.1.1. Kinema

'Kinema' is a Nepali name which has so far been erroneously spelt as 'kenima' (Batra and Millner 1976; Hesselstine 1979; Ramakrishnan 1979; Batra 1986; Campbell-Platt 1987). Although kinema is popular in Nepal, Darjeeling district of West Bengal and Sikkim in India, its antiquity is unknown. It is produced in low lying warm valleys of the area. Kinema is deep fried and used as an adjunct to staples such as rice. Uncooked kinema has a strong ammoniacal odour, but when fried, it has a pleasant nutty flavour (Batra 1986). The method of preparation of kinema was reported briefly by Batra and Millner (1976) and Batra (1986). Whole soya beans are washed, soaked for 24 h, cooked for 2-6 h, cooled to about 40°C, wrapped with broad leaves and left to ferment at 35-45°C for 48-72 h. At the end of fermentation, the beans become covered with a thick, white, mucilaginous coating (Batra 1986). Two rod-shaped, acid-producing bacteria at a level of $2.2-26 \times 10^6$ /g dry weight of kinema were recovered (Batra and Millner 1976; Batra 1986). One of the rods appeared as *Bacillus subtilis* (Batra 1986).

Kinema contains 45-65% moisture, and per 100 g dry matter: 45-55 g protein, 25-30 g fat, 10-15 g carbohydrate, 4-7 g fibre, 5-8 g ash and 2.0-2.1 MJ (490-510 kcal) energy (Campbell-Platt 1987).

Table 2. Traditional fermented foods similar to those common in Darjeeling hills and Sikkim

Name	Area or country	Substrate	Microorganism	Use of food	Reference
Similar to kinema					
Natto	Japan	Soya beans	<i>Bacillus subtilis</i>	Eaten with shoyu or boiled rice	Sakurai (1960); Ohta (1986)
Thua-nao	North Thailand	Soya beans	<i>Bacillus subtilis</i>	Dried paste and chip	Sundhagul <i>et al.</i> (1972)
Tou-shi	China	Soya beans	<i>Bacillus</i> sp.	Seasoning	Yokotsuka (1985)
Tu-su	China	Soya beans	Unknown		Ohta (1986)
Tempe	Indonesia	Soya beans	<i>Rhizopus oligosporus</i>	Fried, meat substitute in soup	Hesseltine <i>et al.</i> (1963); Steinkraus (1983a); Nout and Rombouts (1990)
Daddawa	West Africa	African locust beans	<i>Bacillus subtilis</i> , <i>Bacillus licheniformis</i> , <i>Bacillus pumilus</i>	Condiment	Campbell-Platt (1980); Odunfa (1981, 1985a); Ogbadu and Okagbue (1988)

Name	Area or country	Substrate	Microorganism	Use of food	Reference
Similar to masayura					
Wari	North India and Pakistan	Black gram	<i>Candida krusei</i> , <i>Saccharomyces cerevisiae</i> , <i>Leuconostoc mesenteroides</i> , <i>Lactobacillus fermentum</i> , <i>Streptococcus faecalis</i>	Spicy condiment	Batra and Millner (1974, 1976); Sandhu et al. (1986)
Similar to gundruk					
Sauerkraut	Germany, Switzerland and Central Europe	Cabbage	<i>Leuconostoc mesenteroides</i> , <i>Streptococcus faecalis</i> , <i>Lactobacillus brevis</i> , <i>Lactobacillus plantarum</i> , <i>Pediococcus cerevisiae</i>	Acidic shredded cabbage; used as a side dish	Stamer (1975); Pederson (1979)
Kimchi	Korea	Chinese cabbage, Oriental radish	<i>Leuconostoc mesenteroides</i> , <i>Streptococcus faecalis</i> , <i>Lactobacillus brevis</i> , <i>Lactobacillus plantarum</i> , <i>Pediococcus</i> sp.	Mildly acidic carbonated vegetable; used as a side dish	Mheen et al. (1983)

Name	Area or country	Substrate	Microorganism	Use of food	Reference
Similar to mesu					
Naw-mai-dong	Thailand	Young bamboo shoot	<i>Leuconostoc mesenteroides</i> , <i>Pediococcus cerevisiae</i> , <i>Lactobacillus plantarum</i> , <i>Lactobacillus brevis</i> , <i>Lactobacillus fermentum</i>	Pickle	Dhavises (1972)
Similar to shel roti					
Jalebi	India, Pakistan and Nepal	Wheat flour	<i>Saccharomyces cerevisiae</i> , <i>Saccharomyces bayanus</i> , <i>Hansenula anomala</i> , <i>Streptococcus thermophilus</i> , <i>Lactobacillus bulgaricus</i> , <i>Lactobacillus fermentum</i>	Syrup-filled confection	Batra and Millner (1974); Batra (1981, 1986); Ramakrishnan (1979)
Similar to dahi					
Yoghurt	Worldwide	Milk	<i>Streptococcus thermophilus</i> , <i>Lactobacillus bulgaricus</i>	Soft gel, acidic, non-alcoholic savory	Rašić and Kurmann (1978); Oberman (1985)

Name	Area or country	Substrate	Microorganism	Use of food	Reference
Similar to murcha					
Ragi	Indonesia	Rice flour, herbs and spices	<i>Amylomyces rouxii</i> , <i>Mucor circinelloides</i> , <i>Rhizopus</i> sp., <i>Candida</i> spp., <i>Saccharomycopsis malanga</i> , <i>Pediococcus pentosaceus</i> <i>Saccharomycopsis fibuligera</i>	Starter	Saono <i>et al.</i> (1974); Hesseltine and Ray (1988); Hesseltine and Kurtzman (1990)
Chinese yeast	China and Taiwan	Rice flour, herbs and spices	<i>Amylomyces rouxii</i> , <i>Mucor</i> sp., <i>Rhizopus</i> sp., <i>Saccharomycopsis fibuligera</i> , <i>Saccharomycopsis malanga</i>	Starter	Hesseltine <i>et al.</i> (1988); Hesseltine and Kurtzman (1990)
Bubod	Philippines	Rice flour, herbs and spices	<i>Amylomyces</i> sp., <i>Mucor</i> sp., <i>Rhizopus</i> sp., <i>Saccharomycopsis fibuligera</i> , <i>Saccharomyces cerevisiae</i>	Starter	Tanimura <i>et al.</i> (1977); Del Rosario (1980); Hesseltine and Kurtzman (1990)

Name	Area or country	Substrate	Microorganism	Use of food	Reference
Loogpang	Thailand	Rice flour, herbs and spices	<i>Amylomyces</i> sp., <i>Mucor</i> sp., <i>Absidia</i> sp., <i>Rhizopus</i> sp., <i>Aspergillus</i> sp., <i>Saccharomycopsis fibuligera</i>	Starter	Pichyangkura and Kulprecha (1977); Hesseltine and Kurtzman (1990)
Similar to jnard					
Pachwai or bakhar	India	Rice flour	<i>Hansenula anomala</i> , <i>Endomycopsis fibuligera</i> , <i>Amylomyces rouxii</i> , <i>Mucor fragilis</i> , <i>Rhizopus arrhizus</i>	Alcoholic drink	Batra (1986)
Chiang or lugri	Tibet and Nepal	Barley flour	<i>Saccharomyces cerevisiae</i> , <i>Saccharomyces uvarum</i>	Mildly alcoholic drink	Batra (1986)

2.1.2. Natto

Natto is a popular soya bean fermented food in the Japanese diet. It is gray to tan in colour, and has a strong and persistent unique flavour, sometimes associated with a noticeable odour of ammonia (Steinkraus 1983a; Ohta 1986). Itohiki-natto (sticky natto) is produced by fermenting whole cooked soya beans with *Bacillus subtilis* and accounts for more than the total production of the other two major types of natto. The less common yukiwari-natto is prepared by mixing itohiki-natto with rice koji and salt, and then aging. Hama-natto is prepared by using the koji mould *Aspergillus oryzae* (Kiuchi et al. 1976). Itohiki-natto was traditionally consumed by the Buddhist monks and also by the farmers during winters (Ohta 1986).

Natto is eaten as is with shoyu (main name for soya sauce in Japan) or with mustard, often in breakfast and dinner (Kiuchi et al. 1976; Fukushima 1979) or eaten with boiled rice and often used as a flavouring agent in cooked meat, vegetables and sea foods (Ohta 1986). Japanese domestic soya beans of small and uniform size with white to pale yellow hilum and smooth seed coat are preferred for natto preparation (Ohta 1986).

In the traditional method of natto preparation, soya beans are soaked overnight and boiled until tender. Water is drained off and the beans are allowed to partially air dry over bamboo trays for 20 min. The beans are put into shallow paper containers covered with wax paper, and the containers are stacked one above the other in large wooden boxes, covered with straw-mats, and placed near ovens to ferment at approximately 36°C for one day (Standal 1963).

The straw used as a wrapping material of cooked soya beans, before pine-wood sheet came into use, contained *B. subtilis* (USDA 1958). The use of modern technologies, such as the use of *B. subtilis* as a starter culture was developed after the 1920s (Ohta 1986). The cooked beans are inoculated with spores of *B. subtilis* and tumbled in a barrel until the organisms are well distributed (USDA 1958; Ohta 1986). The beans are wrapped in paper-thin sheets of pine-wood (USDA 1958) or plastic package (Hesseltine and Wang 1967). Polystyrene foams are also in use (Ohta 1986). They are fermented at 40-45°C for 18-20 h (USDA 1958; Hesseltine and Wang 1967; Ohta 1986).

The most favourable conditions for natto production are created by inoculating cooked beans with *B. subtilis* spores 10^8 - 10^9 /ml, equivalent to 0.5-1.0% substrate at 45°C, mixing thoroughly and fermenting at 40-43°C for 6 h (Sakurai 1960). Takahashi and Shimakawa (1976) reported that the best quality natto can be produced by incubating the inoculated beans at 40°C and 85% relative humidity for 12-16 h.

Yabe (1894) was the first to study the microorganisms involved in natto production. Sawamura (1905) identified the fermenting organism as *Bacillus natto* in natto. Gordon *et al.* (1973) considered this species to be a synonym of *Bacillus subtilis* (Ehrenberg) Cohn. However, not all strains of *B. subtilis* are suitable for making good natto (Hesseltine 1983b, 1986). Hayashi (1977) and Ohta (1977) mentioned *Bacillus natto* SB 3010 as the most suitable strain for natto production. The unique feature of *B. natto* is the formation of a sticky viscous material which gives natto its unique

characteristics (Ohta 1986).

Throughout the fermentation period of 18 h, the dry matter and total nitrogen remained fairly constant at 95.5-96.1% and 7.2-7.5%, respectively; water-soluble nitrogen and amino nitrogen increased from 1.26 to 3.13% and 0.07 to 0.6%, respectively; ammonia nitrogen increased from 0.02 to 0.2%; whereas reducing sugars decreased from 13.4 to 11.8%, all expressed on dry matter basis (Sakurai 1960).

Sakurai (1960) compared the starting soya beans (cooked, steamed and surface-dried, but without fermentation) with natto being fermented for 6-8 h. It was found that dry matter and total nitrogen remained fairly constant at 95.5-96.1% and 7.2-7.5%, respectively. Soluble nitrogen increased from 0.89 to 2.88% as a result of proteolytic activity of the organism. Reducing sugars decreased from 13.81 to 11.46% (6 h) and 11.09% (8 h). The ash content decreased slightly from 5.15 to 5.10% (6 h) before an increase to 5.23% (6 h), all expressed on dry matter basis. While the fat content remained relatively constant, total acid (as lactic) increased from 0.42 to 1.17% (8 h) and pH dropped from 6.48 to 6.10 after 6 h and then increased to 6.20 after 8 h. However, Hayashi (1974a,b,c,d) found a 4% increase in total nitrogen in natto over total nitrogen of the raw soya beans. This was because *B. natto* could fix dinitrogen. Natto is rich in essential amino acid content, compared to soya beans (Sano 1961).

The fatty acid composition of natto and soya beans does not differ significantly. The predominant fatty acid in natto is linoleic acid followed by oleic, linolenic and stearic acids (Goto

(1974). The riboflavin content, however, increased after fermentation (Arimoto 1961).

Natto mucilage is composed mainly of an acidic glycopeptide and contains 61.5% sugars, 2.8% hexosamines, 4.1% total nitrogen, 2.9% amino-nitrogen and 20.4% uronic acid (Hayashi *et al.* 1971). Saito *et al.* (1974) found that natto mucin is composed of 58% γ -polyglutamic acid and 40% polysaccharide. Ishikawa *et al.* (1972) examined the characteristic spinnability of a natto mucin solution and found that the mucin contained 22.1% fructan and 77.6% poly-DL-glutamic acid which had high viscoelasticity, and was spinnable due to formation of network structures of randomly coiled poly-DL-glutamic acid through intermolecular H-bondings in the presence of fructan.

The unique flavour of natto was thought to be related to diacetyl content (Obata 1959). Kosuge (1962) identified tetramethyl pyrazine as the flavour component in natto. He also established that some of the free fatty acids, like butyric and isovaleric acids produce an undesirable odour in natto. An ammonia-like odour is directly involved in the quality of natto flavour (Ohta 1986). Soya beans with high carbohydrate content produce less ammonia than those with a lower carbohydrate content. Some of the flavour originates from the hydrolysis of soya protein to peptides and amino acids (Ohta 1986).

Natto has a high nutritional value, improved digestibility and an appreciable amount of certain vitamins, produced as a result of fermentation (Standal 1963; Reddy *et al.* 1982; Steinkraus 1983a; Ohta 1986). Natto is a good source of fibre and free fatty acids (Ohta 1986). Hayashi and Nagao (1975) reported that conversion of

bacterial cells to spores during preservation increases the nutritive value of natto.

Natto contains 50-65% moisture, and per 100 g dry matter: 45-55 g protein, 23-28 g fat, 10-15 g carbohydrate, 4-6 g fibre, 5-10 g ash (higher, if salt added), 2.0 MJ (470-490 kcal) energy, 300 mg Ca, 300 mg P, 1200 mg K, 15 mg Fe, 0.1 mg thiamine, 0.6 mg riboflavin, 1.3 mg niacin, 60 μ g β -carotene and 20 mg vitamin C (Campbell-Platt 1987).

2.1.3. Thua-nao

Thua-nao is a soya bean fermented product common in northern Thailand. Generally available as a dried paste, it is used as a flavouring agent in vegetable dishes. In some areas, the product itself is an item of diet (Sundhagul *et al.* 1972). In the traditional method of its preparation, dry whole soya beans are washed and boiled in excess water for 3-4 h till they can be crushed between fingers. Excess water is drained off and the cooked beans are transferred to a bamboo basket lined with banana leaves. The basket is covered with banana leaves. The beans are left at room temperature for 3-4 days to undergo natural fermentation, and are considered properly fermented when they are covered with a sticky, viscous material, accompanied by pungent odour of ammonia replacing the beany flavour. The beans change from light brownish yellow to greenish brown colour (Sundhagul *et al.* 1972). After fermentation, the raw thua-nao is mashed lightly into paste and added with salt, garlic, onion and red pepper. The paste is wrapped in banana leaves and cooked by steaming before eating (Sundhagul *et al.* 1972).

The cooked thua-nao paste, for its high moisture content, is kept for only about two days at room temperature. On the other hand, thua-nao chips can be prepared by cutting raw thua-nao paste into thin chips and then sun-drying, and kept for several months (Sundhagul *et al.* 1972).

The fermenting organism for thua-nao has been identified as *Bacillus subtilis*. The initial bacterial load of 10^3 cells/g cooked beans was increased to 10^{10} cells/g thua-nao. The increase was rapid during the first two days. During fermentation, the pH increased from 6.3 to 8.6 in the second day and remained relatively unchanged afterwards. The moisture level at 62% remained relatively constant. Chemical determination of thua-nao paste and chips showed that they had high protein and fat contents. The protein contents were 16.9 and 36.8%, and the fat contents were 7.4 and 14.8% for paste and chips, respectively (Sundhagul *et al.* 1972).

A low cost, protein-rich food, called 'ferm-soya-mix' in powder form, ready to eat with long shelf-life under normal conditions has been developed by blending thua-nao powder with flavouring agents and a small proportion of high grade fish meat (Sundhagul *et al.* 1973).

2.1.4. Tou-shi

Tou-shi, shi-tou-shi or shi is a soya bean fermented food commonly consumed in China. In the traditional method of its preparation, soya beans (yellow or black) are cooked, cooled, placed in a pile on a straw-mat, covered with straw and fermented at 25-30°C for 1-2 days. Tou-shi is mixed with minced ginger and salt, and then tightly packed into jars. After aging for one week, they are ready

for consumption (Yokotsuka 1985).

On the basis of microorganisms employed, tou-shi can be classified as *Aspergillus oryzae*, *Mucor* or *Bacillus* type (Yokotsuka 1985).

2.1.5. Tempe

'Tempe kedele', usually referred to as 'tempe', is one of the most important traditional fermented soya bean foods in Indonesia. Fresh tempe has a clean, mushroomy or nutty odour (Nout and Rombouts 1990). It is not consumed raw, but heated to develop meat-like flavour by frying spiced and salted slices in oil, by boiling with coconut milk in soups, by stewing, by roasting spiced kebabs, and in peppered ground pastes (Shurtleff and Aoyagi 1979; Soewito 1985). On deep frying, the flavour of tempe becomes nut-like and peppery, due to the presence of free fatty acids (Steinkraus 1983a).

Most cultivars of yellow-seeded soya beans are suitable for tempe, in contrast to black-seeded ones (Sharma and Sarbhoy 1984). Traditionally, soaked, hand-dehulled and briefly boiled beans are inoculated with small pieces of tempe from a previous fermentation, wrapped in banana leaves which also serve as a source of inoculum, and left at room temperature for 1-2 days (Wang 1986b).

At present, most wet dehulling of soya beans is mechanized in the Indonesian traditional process, using simple electric-driven disc dehullers. After removal of hulls, hydration is carried out by autoclaving, boiling, steaming or by overnight soaking (Nout and Rombouts 1990). Addition of lactic (<0.5%) or acetic (<0.25%) acid during hydration to control microbial spoilage has been

suggested (Usmani and Noorani 1986; Wadud *et al.* 1988). Emphasis has been given to the importance of acid fermentation or artificially acidifying the beans, because the mould is proteolytic, and deamination following hydrolysis releases ammonia, causing the pH to rise. Above pH 7.0, sufficient free ammonia is released to kill the mould and a lower initial pH allows a longer fermentation time before ammonia is liberated (Steinkraus 1983a).

During the traditional tempe manufacturing process, spontaneous and uncontrolled fermentations of soya beans take place during the soaking period prior to mould fermentation. Acidifying the beans during soaking to pH ≤ 4.30 yields tempe of good quality in which bacilli and Enterobacteriaceae could not be detected. The acidification during soaking can be controlled by recycling part of the soak water from a previous batch as an inoculum, contributing to the shelf-life and safety of tempe (Nout *et al.* 1987).

Cooking by steaming for at least 30 min at 100°C (Djien and Hesseltine 1979) or by boiling in excess water for 2-3 h (Winarno and Reddy 1986) serves the purpose of partial cooking which facilitates fungal penetration and human digestion (Nout and Rombouts 1990). Partial cooking of soya beans destroys trypsin inhibitors (Albrecht *et al.* 1966), inactivates some undesirable factors such as phytic acid (Chang *et al.* 1977; Toma and Tabekhia 1974) and flatus-causing oligosaccharides (Wang *et al.* 1979), leaches out a heat-stable and water-soluble mould inhibitor (Wang and Hesseltine 1979; Djien and Hesseltine 1979), destroys contaminating bacteria that interfere with fermentation, releases

some of the nutrients required for mould growth (Steinkraus 1983a), and destroys the bitter soya taste (Nout et al. 1985).

Following cooking, the beans should have no excess moisture on them, because the presence of free water on the cotyledons favours bacterial growth and spoilage during or following the mould fermentation (Steinkraus 1983a). The use of basket centrifuges for removal of boiling water from the cotyledons has been advised by Shurtleff and Aoyagi (1980). Addition of approximately 2% w/w maize starch, rice flour or cassava starch helps to absorb the remaining moisture, stimulates fungal growth and results in better tempe firmness (Nout and Rombouts 1990).

The inoculum for tempe fermentation can be obtained from dried and pulverized tempe of previous batch ('tempe-to-tempe'), mould grown and air dried on leaves of *Hibiscus* spp., *Tectona grandis*, *Bambusa* sp. or *Musa paradisiaca*, locally referred to as 'usar' or 'laru' (Djien and Hesseltine 1979) sold on Indonesian markets or ragi (2.5 cm in diameter) containing the tempe mould and a variety of microorganisms also sold on Indonesian markets.

Studies carried out by Steinkraus et al. (1960) and Hesseltine et al. (1963) resulted in a pure culture fermentation. The most popular strain is *Rhizopus oligosporus* NRRL 2710 which grows at 30-42°C (Steinkraus 1983a; Hesseltine 1985a). But use of pure culture starters for large scale industrial purpose is too expensive and time-consuming (Djien 1985). Therefore, semi-pure culture starters are prepared by growing a pure culture of *Rhizopus* strain on traditionally cooked or steamed substrate, mostly rice (Djien 1985) or soya beans (Usmani and Noorani 1986). The dry

starters contain mould as well as bacteria (Djien 1985). Instead of leaving it to chance, attempts were made to prepare mixed culture starters with simultaneous growth of lactic acid bacteria and *R. oligosporus* by adding 1% sour soak water to the boiled substrate. The resulting tempe was of superior quality (Tüncel et al. 1989). Mixed pure cultures of *R. oligosporus* and *Klebsiella pneumoniae* are used to produce vitamin B₁₂-containing tempe (Areekul et al. 1990).

Large leaves, used traditionally, are excellent for wrapping beans for fermentation (Steinkraus 1983a). But, rough-surfaced leaves result in tempe with irregular surfaces, because tempe takes the shape of its fermentation container. Smooth polythene sheets, metallic or hard plastic boxes give tempe with straight edges and smooth shiny surfaces. An interesting development was the use of plastic bags or tubes perforated at 0.2-1,3 cm intervals to allow access of oxygen for the mould (Martinelli and Hesseltine 1964). Plastic bags for tempe fermentation have also been widely adopted for use in Indonesia (Wang and Hesseltine 1979). Steinkraus et al. (1960) used covered stainless steel cake pans.

Incubation takes 80-22 h at 25-37°C, respectively. The higher the incubation temperature, the more rapidly *R. oligosporus* grows (Martinelli and Hesseltine 1964). The optimum relative humidity during tempe preparation was reported as 60-65% (Usmani and Noorani 1986), 75% (Wadud et al. 1988) and 90% (Steinkraus 1985).

As soon as the bean cotyledons are overgrown completely by the mould and knitted into a compact cake, tempe is harvested and cut into cubes (2.5 cm x 2.5 cm). It is then directly transported

to the market or preserved by boiling in brine, steaming, canning, dehydration or deep frying (Djien and Hesseltine 1979; Winarno 1985; Winarno and Reddy 1986).

As the mould begins to grow rapidly during tempe fermentation, the temperature of the fermenting beans rises from 5 to 7°C above the incubation temperature. As a result of protein metabolism, pH increases from 4.5 (0 h) to 6.0 (26 h at 28°C, 18 h at 38°C) and 7.0 (48 h at 28°C, 30 h at 38°C), leveling off towards pH 7.5 to 8.0. During fermentation, there is increase in total soluble solids, soluble nitrogen and free amino acids, whereas total nitrogen remains fairly constant (Steinkraus *et al.* 1960; Wang and Hesseltine 1966; Wang *et al.* 1968).

Rhizopus oligosporus produces two proteolytic enzyme systems, one with an optimum activity at pH 3.0 and the other at 5.5, both having maximum activity at a temperature of 50-55°C; maximum proteolytic activity was attained at 72-96 h at 32°C (Wang and Hesseltine 1965).

Rhizopus oligosporus possesses a strong lipolytic activity, hydrolyzing over one-third of neutral fat of soya beans after 72 h fermentation at 37°C. Lipolysis yields predominantly linoleic acid, besides oleic, palmitic, linolenic and stearic acids (Wagenknecht *et al.* 1961). The free fatty acids, particularly oleic, linoleic and linolenic acids are associated with non-specific antitryptic activity (Winarno and Reddy 1986). *Rhizopus oligosporus* derives much of its energy from oleic acid (Nout and Rombouts 1990). This was supported by findings of Paredes-Lopez *et al.* (1987) who reported a 50% reduction of oleic acid in bean tempe.

Carbohydrates of soya beans, especially raffinose and stachyose cause flatulence (Nout and Rombouts 1990). During fermentation, there is rapid removal of hexoses and slow hydrolysis of stachyose (Shallenberger *et al.* 1967). Total flatus factors are reduced from 16.5 to 2,0 mg/g soya beans (Winarno and Reddy 1986). Protein-bound starch decreases the digestibility of soya protein; during prolonged fermentation (48-72 h), starch decreases from 0.4 to 0.1% (dry weight) with the formation of some unidentified carbohydrates (van der Riet *et al.* 1987).

Tempe has been reported to contain nutritionally important amount of vitamin B₁₂ (Steinkraus *et al.* 1961; van Veen and Steinkraus 1970; Liem *et al.* 1977; Winarno 1979; Truesdell *et al.* 1987). Except for thiamine which was reduced by approximately 50%, all other vitamins including riboflavin, nicotinic acid, pantothenic acid, pyridoxine, folic acid, cyanocobalamine and biotin increased significantly (Shurtleff and Aoyagi 1979; Okada *et al.* 1983; Murata 1985).

Murata (1977) attributed the improved nutritive value of tempe to stabilization of the oil by antioxidants produced during the fermentation and synthesis of B vitamins. Steinkraus (1983a) observed that stored tempe does not develop rancidity because of its content of 6,7,4'-trihydroxyisoflavone, an antioxidant produced by the mould.

According to Whitaker (1987), the beany flavour would be released from the proteins to which they are bound as a result of proteolysis. Flavour components of the boiled soya beans included mannitol, esters (ethyl palmitate and ethyl linoleate) and free

fatty acids (palmitic, stearic, oleic, linoleic, linolenic). Tempe prepared at 38°C had a stronger odour than that prepared at 31°C. The flavour of tempe prepared at 31°C included the original soya bean components and newly formed 3-methylbutanol, acetoin, acetic acid, methylcarbinol, 2,3-butanediol and isovaleric acid (Moroe 1985).

Rhizopus oligosporus NRRL 2710 produced an antibiotic active against a number of Gram positive bacteria including *Staphylococcus aureus* and *Bacillus subtilis*. The only Gram negative bacterium sensitive to the antibiotic was *Klebsiella pneumoniae* (Wang et al. 1969).

Work on protein efficiency ratio (PER) and the digestibility of tempe (Wang 1986b; Zamora and Veum 1988; Agosin et al. 1989) confirmed that rats do not utilize protein from tempe any better than from cooked substrate. However, Giriya Bai et al. (1975) and Winarno and Reddy (1986) reported that mixed soya beans-groundnut tempe gave better net protein utilization (NPU) and PER than soya bean protein .

Tempe-like products could also be made from wheat, rice, other cereal grains and their various combinations (Hesseltine et al. 1967).

Tempe contains 25-65% moisture, and per 100 g dry matter: 45-55 g protein, 15-25 g fat, 15-25 g carbohydrate, 3-7 g fibre, 5-10 g ash, 1.8-1.9 MJ (430-460 kcal) energy, 400 mg Ca, 400 mg P, 25 mg Fe, 0.4 mg thiamine, 0.7 mg riboflavin, 6 mg niacin, 0.3 mg pantothenic acid, trace vitamin B₁₂ and 50 µg vitamin A (Campbell-Platt 1987).

2.1.6. Daddawa

Daddawa or iru is a fermented food produced by natural fermentation of the cotyledons of African locust bean (*Parkia biglobosa* Welw. ex Oliv.), a perennial tree legume, commonly consumed in the Savannah regions of West Africa (Campbell-Platt 1980; Eka 1980; Odunfa 1985a). Daddawa, a protein and fat-rich flavoursome ingredient, is used as a good condiment and eaten with sorghum or millet-based dumplings and porridges (Campbell-Platt 1980; Odunfa 1986).

Daddawa preparation is still a traditional family art done in homes. In the traditional method of its preparation, the dried pods are boiled for 12-24 h to soften the tough testa and cotyledons. The seeds are put in a mortar, pressed with feet to remove the softened testa; sand or other abrasive agents may be added. The cotyledons are washed and boiled again for 1-2 h. Excess water is drained off. Seeds are spread in calabash trays in layers of about 10 cm deep or in a hole in the ground. They are left at 25-35°C for 3-4 days. Wood ash may be mixed to reduce the odour. Sometimes, millet flour may be added. The fermented bean mass is sun-dried, and then used loose, or shaped into balls or pyramids and stored in the traditional earthenware pots (Campbell-Platt 1980; Odunfa 1981, 1985a, 1986).

Odunfa and Adewuyi (1985) studied the optimization of process conditions for daddawa production and found the optimal time/temperature for fermentation were 36 h at 35°C and 48 h at 40°C.

Osinowo et al. (1990) improved the traditional method of daddawa fermentation by cooking beans in pressure cooker for 75-90

min to remove seed coats, cleaning, washing, further cooking with the addition of starter culture for 1 h, placing in flat calabash and fermenting at 30°C for 18 h.

The presence of *Bacillus subtilis*, *B. licheniformis* and *Staphylococcus* spp. was reported in daddawa (Odunfa 1981, 1986). Antai and Ibrahim (1986) reported the presence of *Leuconostoc mesenteroides* and *L. dextranicus* in almost equal proportion with the *Bacillus* spp. in daddawa. However, Ogbadu and Okagbue (1988) could not find any of these lactics during daddawa production. They found that the species of *Bacillus* responsible for daddawa production were variable, and reported *B. subtilis*, *B. pumilus* and *B. licheniformis* from six separate fermentations. Osinowo *et al.* (1990) reported *B. subtilis*, *B. cereus*, *Pseudomonas aeruginosa* and *Enterobacter aerogenes* from daddawa.

During fermentation, the temperature and pH of the beans increased from 25°C and 7.0 at 0 h to 45°C and 8.1 at 36 h, respectively (Odunfa 1981). The fatty acids in both unfermented and fermented beans were linoleic, oleic, stearic, palmitic and a trace of arachidonic acids. The major fatty acid was linoleic acid which is an essential fatty acid (Odunfa and Adesomoju 1986). The amino acid pattern of fermented *Parkia* beans was similar to that of raw beans, with a small decrease in essential sulphur-containing amino acids and large decrease in the non-essential aspartic and glutamic acids (Fetuga *et al.* 1973). The quantities of the flatus-forming oligosaccharides decreased significantly during the first 24 h of fermentation and this decrease was attributed to the activities of α - and β -galactosidase which hydrolyzed the oligosaccharides to reducing sugars (Odunfa 1983). Thiamine and

riboflavin content increased during fermentation (Platt 1964; Leung *et al.* 1968; Eka 1980).

Daddawa contains 20-50% moisture, and per 100 g dry matter: 40-45 g protein, 30-40 g fat, 10-15 g carbohydrate, 3-7 g fibre, 3-6 g ash, 2.1-2.3 MJ (500-600 kcal) energy, 300 mg Ca, 550 mg P, 40 mg Fe, 0.05 mg thiamine, 0.6 mg riboflavin, 2 mg niacin and 0.9 μ g folic acid (Campbell-Platt 1987).

2.1.7. Wari

Waries or Punjabi waries are fermented black gram products, common in northern India and Pakistan. These are dried, hollow, brittle, spicy and friable balls, 3-8 cm in diameter and 15-40 g in weight. Waries are used as condiments or adjuncts in cooking vegetables, legumes or rice (Batra 1986; Soni and Sandhu 1990).

In the traditional method of wari preparation, black gram [*Vigna mungo*(L.) Hepper] dhals are soaked in water for 6-12 h, dewatered, dehulled and ground on a stone martar into a smooth, mucilaginous paste. The dough is mixed with inoculum from a previous batch, salt and typical spices like asafoetida (*Ferula foetida* Regel), caraway (*Carum carvi* L.), cardamom [*Elettaria cardomomum* (L.) Moton], clove [*Syzygium aromaticum* (L.) Merr. and Perry], fenugreek (*Trigonella foenum-groecum* L.), ginger (*Zingiber officinale* Rosc.) and red pepper (*Capsicum annuum* L.). The mixture is allowed to ferment at room temperature for 1-3 days and hand-moulded into balls. After air-drying for 2-8 days on bamboo or palm mats, waries are turned over for further drying (Batra and Millner 1976; Batra 1981; Soni and Sandhu 1990).

Batra and Millner (1974, 1976) isolated two types of yeasts including *Candida krusei* and *Saccharomyces cerevisiae* from waries. Later on, although a wide variety of yeasts and lactic acid bacteria were found to be associated with waries, only the combination of *Hansenula* sp. and *Leuconostoc mesenteroides* was found responsible for their production (Batra 1981, 1986).

Sandhu and Soni (1989) observed the occurrence of bacteria (10^9 - 10^{12} /g) in all the market and laboratory-made samples, but only 55% of the samples contained yeasts (0 - 10^7 /g). *Leuconostoc mesenteroides* was most abundant and present in all the market samples, followed by *Streptococcus faecalis*, *Lactobacillus fermentum* and *Bacillus subtilis*. *Saccharomyces cerevisiae* and *Pichia membranaefaciens* were the most abundant yeasts, found in all the positive samples, followed by *Candida vartiovaarai*, *Kluyveromyces marxianus*, *Trichosporon beigelii*, *Candida krusei* and *Hansenula anomala*. Laboratory-made samples were found to contain comparatively higher bacterial load (10^{10} - 10^{12} /g) while less yeast load (0 - 10^6 /g) in 45% of the samples.

The microbial load of 1.3×10^{10} /g unfermented dough increased to 6.5×10^{12} /g at the end of fermentation. Among the bacteria, *Leuconostoc mesenteroides*, *Lactobacillus delbrueckii*, *Lactobacillus fermentum*, *Bacillus subtilis* and *Flavobacter* spp., and among the yeasts, *Trichosporon beigelii*, *Saccharomyces cerevisiae*, *Candida krusei*, *Pichia membranaefaciens* and *Hansenula anomala* predominated the initial stages of fermentation. With the progress in fermentation, most of the microorganisms, except *Leuconostoc mesenteroides*, *Lactobacillus fermentum*, *Saccharomyces cerevisiae*

and *Trichosporon beigelii*, disappeared. There was the production of acid and gas resulting in the fall in pH from 5.65 to 3.20 and rise in volume from 200 to 420 ml. Fermentations brought about an increase in total acids from 0.50 to 1.50%, soluble solids from 7.8 to 14.7%, non-protein nitrogen from 0.20 to 0.68%, soluble nitrogen from 0.95 to 1.50%, free amino acids from 9.79 to 45.15 mg/g and proteolytic activity from 4.82 to 6.04 IU/g. On the other hand, the level of reducing sugars and soluble protein decreased from 13.69 to 4.34 mg/g and 50.52 to 17.40 mg/g, respectively. Amylase activity increased initially, but declined thereafter. Wari fermentation also brought about an appreciable rise in water-soluble B vitamins including thiamine, riboflavin and cyanocobalamine (Sandhu et al. 1986; Sandhu and Soni 1989; Soni and Sandhu 1990).

2.2. FERMENTED VEGETABLES

2.2.1. Gundruk

Gundruk, a non-salted and fermented leafy vegetable product, has been one of the major appetizers for the people of Nepal since a long time back (Karki et al. 1983d). It is cooked in water and served as a side dish (Dietz 1984).

In the traditional method of its preparation, fresh leaves of mustard [*Brassica juncea* (L.) Czern.], radish (*Raphanus sativus* L.), cauliflower (*Brassica oleracea* L. var. *botrytis* L.) and rape (*Brassica campestris* L.) are left for wilting for 2-3 days. The leaves are shredded; pressed into an earthen jar and covered with lukewarm (30-35°C) water. After fermentation at 16-20°C for 5-7 days, the leaves are removed from the jar and sun-dried (Karki et al. 1983d; Karki 1986).

In the samples of gundruk from Nepal, the microflora, represented by lactic acid bacteria, contained *Lactobacillus plantarum*, *L. casei* ssp. *casei*, *L. casei* ssp. *pseudoplanatarum*, *L. cellobiosus* and *Pediococcus pentosaceus*. During fermentation, heterofermentative *Lactobacillus cellobiosus*, instead of *Leuconostoc mesenteroides* as in other fermented vegetable products, initiates the fermentation and is followed by homofermentative *Pediococcus pentosaceus* and finally *Lactobacillus plantarum* (Karki et al. 1983d).

The pH and acidity (as lactic) in gundruk were 4.0-4.3 and 0.8-1.0%, respectively (Karki et al. 1983d). In gundruk, almost 90% of the organic acids consisted of lactic and acetic acids. Besides, citric and malic acids were found in lower concentrations (Karki 1986). The level of palmitic, oleic, linoleic and linolenic acids was much higher in mustard leaf gundruk compared to those in the unfermented vegetables (Karki et al. 1983c). In mustard gundruk, free amino acids, particularly glutamic acid, alanine, leucine, lysine and threonine remarkably increased with the corresponding decrease in asparagine, glutamine, histidine and arginine, indicating the influence of fermentation. Proline content in mustard vegetable was greater than that in cauliflower gundruk or mustard gundruk. This may be due to the wilting of vegetables prior to fermentation (Karki et al. 1983b).

The main flavour components developed during the fermentation of mustard leaves are cyanides (15.7%), isothiocyanates (8.5%) followed by alcohols (12.3%) and esters (4.1%). Phenylacetaldehyde (6.4%) was the only aldehyde identified in mustard leaf gundruk

(Karki et al. 1983a).

According to Dietz (1984), vitamin A is lost during sun-drying of gundruk.

2.2.2. Sauerkraut

Sauerkraut or sauerkohl is a German term for 'sour cabbage', which is generally prepared from shredded white cabbage. It is eaten with main meals in Germany, Switzerland, Central Europe, USA, Canada and USSR (Pederson 1979; Campbell-Platt 1987).

For the preparation of sauerkraut, cabbage (*Brassica oleracea* L. var. *capitata*) is trimmed, washed, shredded (3-5 mm x 5-7 cm), placed in barrels with 2.0-2.5% salt, distributed evenly and packed tightly in layers, covered, sealed and allowed to ferment at 16-22°C for 1-2 weeks, followed by gradual reduction in temperature to 0-5°C at the end of one month (Stamer 1975; Frazier and Westhoff 1978; Pederson 1979; Steinkraus 1983b; Vaughn 1985).

Pederson (1930a,b) determined the sequence of microorganisms that develop in a typical sauerkraut fermentation. Subsequent studies by Pederson and Albury (1954, 1969) and Stamer et al. (1971) established that *Leuconostoc mesenteroides* initiates fermentation in the shredded cabbage over a wide range of temperature and salt concentration, producing carbon dioxide and lactic acid, followed by predominance of *Lactobacillus brevis* and *Lactobacillus plantarum*. If the fermentation temperature is higher, *Pediococcus cerevisiae* develops and contributes to acid production. While low salt concentration (1.0%) favours the growth of heterofermentative lactics including *Leuconostoc mesenteroides* and

Lactobacillus brevis, a higher concentration (3.5%) of salt favours the growth of homofermentative lactics including *Pediococcus cerevisiae* and *Lactobacillus plantarum*.

The fermentation was very slow at 7.5°C, producing 0.8-0.9% acidity (as lactic) in a month, but rapid at 23°C, producing 1.0-1.5% acidity in 8-10 days and more rapid at 32°C, producing 1.8-2.0% acidity in 8-10 days (Pederson and Albury 1969). Higher temperature may result in inferior quality and dark kraut (Pederson 1979). The optimum temperature of about 18°C at 2.25% salt was recorded for sauerkraut fermentation (Parmele *et al.* 1927; Marten *et al.* 1929; Pederson and Albury 1969). During fermentation, carbohydrates are converted to lactic and acetic acids, ethanol, carbon dioxide, mannitol and dextran (Pederson 1979).

Mukherjee *et al.* (1977) observed the loss of total nitrogen from 0.23% (30 days) to 0.12% (120 days), loss of total ash, and gradual increase of crude fibre during sauerkraut production.

The major amount of the volatiles in sauerkraut is accounted for by acetal, isoamyl alcohol, n-hexanol, ethyl lactate, cis-hex-3-ene-1-ol and allyl isothiocyanate. Only the two latter compounds have been identified as major constituents of fresh cabbage (Lee *et al.* 1976).

Lactobacillus brevis produces a red pigment under certain conditions which may result in discolouration or darkening of sauerkraut (Stamer *et al.* 1973). The growth of pigmented yeast may be the cause of kraut defect or 'pink kraut' (Brunkow *et al.* 1925). In fact, anaerobiosis helps to eliminate aerobic growth of moulds and yeasts in sauerkraut (Pederson and Albury 1969).

Sauerkraut contains 35-45% moisture, and per 100 g dry matter: 3-5 g protein, trace amount of fat, 15-20 g carbohydrate, 25-30 g fibre, 35-45 g ash, 15-25 g NaCl, 0.3-0.4 MJ (70-100 kcal) energy, 150 mg Ca, 2 mg Fe, 0.1 mg thiamine, 0.15 mg riboflavin, 0.7 mg niacin, 50 µg carotene and 50-70 mg ascorbic acid (Campbell-Platt 1987).

2.2.3. Kimchi

Kimchi is the general name given to a group of fermented acid vegetable foods with a long tradition in Korea. More specific names are used for pickled vegetables depending on the raw material, processing method, season and locality. It is a side dish served along with cooked rice. Kimchi is closely related to sauerkraut, but differs in having less acid and being carbonated (Mheen *et al.* 1977; Lee 1986).

For the preparation of kimchi, Oriental radish (*Raphanus sativus* L.), Chinese cabbage (*Brassica chinensis*), cucumber (*Cucumis sativus* L.) or other vegetables are mixed with small amount of onion, chilli, pepper, garlic, ginger and 4-6% salt or brine. The mixture is packed into a large earthenware vessel. Fish, shrimps or oysters may be added and fermented at 10-18°C for 5-20 days (Mheen *et al.* 1977; Lee 1986).

Kimchi contains *Leuconostoc mesenteroides*, *Streptococcus faecalis*, *Lactobacillus brevis*, *Lactobacillus plantarum* and *Pediococcus cerevisiae* (Kim and Whang 1959; Kim and Chun 1966; Mheen and Kwon 1979). Whang *et al.* (1960) isolated *Achromobacter*, *Flavobacterium* and *Pseudomonas* spp. from kimchi. Ha (1960) observed

that few yeasts and moulds appear in the later stages of fermentation, causing softening of the product. Pathogenic bacteria present on the ingredients disappear during fermentation (Soh 1960; Chung *et al.* 1967).

At the time of fermentation, the initial pH of 5.5-5.8 falls to pH 4.2-4.5 (Song *et al.* 1966). The optimum acidity of kimchi is 0.4-0.8% (as lactic), while higher acidity makes it unacceptable (Lee and Yang 1970; Mheen *et al.* 1977). Kimchi produced at 6-7°C contained more lactic and succinic acids but less oxalic, malic, tartaric, malonic, maleic and glycolic acids than that produced at 22-23°C (Kim and Rhee 1975). Lee and Lee (1965) reported a decrease in reducing sugars during fermentation.

Using a 3% salt concentration, the optimum period of fermentation was one day at 30°C, 2-3 days at 20°C, 12-15 days at 10°C, and 30-60 days at 5°C (Yu and Chung 1974; Mheen *et al.* 1977). Vitamins including thiamine, riboflavin, cyanocobalamine and niacin reached their highest levels (twice the initial level) when kimchi had the most palatable taste, and decreased when kimchi became too sour (Lee *et al.* 1960). Kimchi produced following inoculation with *Propionibacterium freudenreichii* van Niel ssp. *shermanii* contained 102 µg cyanocobalamine, whereas non-inoculated fermentation had 47 µg cyanocobalamine per 100 g substrate (Ro *et al.* 1979). Vitamin C and carotene decrease upon ripening (Lee *et al.* 1960; Lee and Lee 1965; Song *et al.* 1966).

Kimchi contains 75-95% moisture, and per 100 g dry matter: 10-30 g protein, 3-10 g fat, 30-50 g carbohydrate, 5-10 g fibre, 10-20 g ash, 1.0-1.4 MJ (250-330 kcal) energy, 20-300 mg Ca, 250-600 mg P, 2-11 mg Fe, 0.15-0.7 mg thiamine, 0.2-1.0 mg riboflavin, 3-40

mg niacin, 100-300 µg cyanocobalamine and 75-450 mg vitamin C (Campbell-Platt 1987).

2.2.4. Naw-mai-dong

Naw-mai-dong, the pickle obtained by fermenting young shoots of bamboo [*Bambusa arundinacea* (Retz.) Roxb.] is common in Thailand. Those of the sweeter species, such as *Bambusa burmanica* Gamble and *Dendrocalamus asper* Back. are also used as raw materials (Boon-Long 1986).

In the traditional method of its preparation, bamboo shoots are boiled in water and the bitter liquor is discarded. They are then sliced (2-3 mm x 1.5 cm), mixed with 2% salt, packed into a narrow-mouthed earthen jar, weighted down and fermented at room temperature for 3-4 weeks (Dhavises 1972; Yanasugondha 1977; Boon-Long 1986).

Naw-mai-dong contained 1-1.2% lactic acid (Boon-Long 1986). *Pediococcus cerevisiae* predominates at the early stages of fermentation. *Lactobacillus plantarum* comes to predominate after 6 h and *Lactobacillus brevis* predominates at the final stage (Dhavises 1972).

2.3. FERMENTED CEREAL PREPARATION

2.3.1. Jalebi

Jalebies are pretzel-like syrup-filled confections, prepared from deep-fried fermented wheat-flour batter. These are consumed throughout India, Nepal and Pakistan (Batra and Millner 1974, 1986; Ramakrishnan 1979). They have been known in these areas since 1450 A.D. and are probably of Arabic or Persian origin (Gode 1943).

Jalebies are prepared by mixing wheat flour with dahi (curd), adding water in it and leaving overnight at room temperature. The thick leavened batter is squeezed through an embroidered hole (about 4 mm in diameter) in thick and durable cotton cloth, and deposited as continuous spirals into hot fat. After about one min, when the spirals become light brown, these are removed from fat with a sieved spatula. Excess fat is drained away, and the jalebies are immediately immersed into sugar syrup for 1-2 min. Often rose (*Rosa indica* Lour.) or kewda (*Pandanus tectorius* Soland. ex. Parkinson) water and orange food colour are added to the syrup (Ramakrishnan 1977, 1979; Batra 1981, 1986).

Ramakrishnan (1977, 1979) reported the presence of *Lactobacillus fermentum* (6×10^8 /g), *L. buchneri* (3.2×10^8 /g), *Streptococcus lactis* (6×10^8 /g), *S. faecalis* (6×10^8 /g) and *Saccharomyces* sp. in fermented jalebi batter. But, Batra (1981, 1986) found *Lactobacillus bulgaricus*, *Streptococcus thermophilus*, *S. faecalis*, *Saccharomyces bayanus*, *Saccharomyces cerevisiae* and *Hansenula anomala* in fermented jalebi batter. During fermentation at 28°C, the bacterial and yeast counts increased from 3.26×10^5 to 12.6×10^6 /g and 9.4×10^4 to 6×10^6 /g, respectively. At 19°C, while the bacterial count was lowered to 1×10^6 /g, there was no change in the count of yeasts.

During fermentation, the pH decreases from 4.4 to 3.3 and the volume of the batter increases by 9%. Amino nitrogen and free sugar contents decrease during fermentation (Ramakrishnan 1977, 1979).

Jalebi contains 32-38% moisture, and per 100 g dry matter:

4-7 g protein, 15-20 g fat, 75-78 g carbohydrate, 2-4 g fibre, 2-3 g ash, 1.9-2.0 MJ (460-480 kcal) energy, 2 mg Na, 90 mg K, 70 mg Ca, 10 mg Mg, 1 mg Fe, 0.1 mg Cu, 0.5 mg Zn, 0.17 mg thiamine, 0.03 mg riboflavin, 2.0 mg niacin, 14 µg folic acid, retinol, carotene, vitamin C and D (Campbell-Platt 1987).

2.4 FERMENTED DAIRY PRODUCTS

2.4.1. Dahi

Dahi, a major adjunct to the daily diet in India, Pakistan, Nepal, Bangladesh and Sri Lanka, is the result of action of lactic acid bacteria on cow's or buffalo's milk. It resembles plain yoghurt in appearance and consistency, and differs in having less acidity (Batra and Millner 1976; Mital 1977; Shuaib and Azmey 1977; Ekmon and Nagodawithana 1977).

In the traditional method of its preparation, milk is boiled, cooled, inoculated with previous batch of dahi and kept at ambient temperature for 8-12 h for setting (Verma and Mathur 1986).

Laxminarayana et al. (1952a,b) observed that dahi from north India is firm and sweet to mildly sour in taste, with a preponderance of streptococci over lactobacilli, whereas dahi from south India is soft and acidic, with more lactobacilli than streptococci. In the eastern part of India, misti dahi (sweetened dahi or payodhi) is very popular (Ghosh and Rajorhia 1987, 1990).

Laxminarayana et al. (1952b), Ranganathan et al. (1964), and Ramakrishnan (1979) isolated *Lactobacillus bulgaricus*, *L. acidophilus*, *L. helveticus*, *L. casei*, *L. brevis*, *Streptococcus thermophilus*,

S. lactis, *S. cremoris* and *S. faecalis* from dahi.

Ranganathan *et al.* (1964) observed that in the microflora of dahi *S. thermophilus* constitutes 50% of total streptococci with *S. faecalis* and *S. lactis* next most numerous, and *L. bulgaricus* constitutes 70% with *L. casei* and *L. brevis* next most numerous. A mixed culture of *S. thermophilus* and *L. bulgaricus* produced greater amount of acid than mixed culture of *S. thermophilus*, *S. lactis* *ssp. diacetylactis* and *S. lactis* (Sharma and Jain 1975). However, *S. lactis* *ssp. diacetylactis* imparted desirable flavour to dahi by producing higher amount of diacetyl and volatile acid than *S. thermophilus* and *S. cremoris* (Baisya and Bose 1975).

Ghosh and Rajorhia (1990) found that a mixture of various strains of *S. lactis*, *S. diacetylactis*, *S. cremoris* and *Leuconostoc* sp. was most appropriate for production of misti dahi from buffalo's milk containing 18% milk solids and 14% sucrose.

Batra and Millner (1976) isolated yeasts from dahi of Punjab and identified them as *Candida krusei*, *Trichosporon* sp. and *Torulopsis* sp.

A good quality dahi has a pH 4.6-5.0 (Rao and Dastur 1955) and acidity 0.8-1.0% as lactic (Srinivasan and Banerjee 1946). During fermentation, there is increase in non-protein nitrogen and dialyzable nitrogen, but decrease in protein nitrogen and ammonia nitrogen (Venkatappaiah and Basu 1956; Verma and Mathur 1986).

Rao and Basu (1962) found that a mixed culture of *L. bulgaricus* and *L. cremoris* decreased thiamine, riboflavin and nicotinic acid content, whereas single cultures of *S. lactis* and *S. cremoris* raised the thiamine concentration from 2 to 20% over that of milk

during dahi fermentation. Boman and Dalal (1956) observed the increase of riboflavin, folic and folinic acid content during dahi fermentation.

Dahi contains 85-88% moisture, 3.2-3.4% protein, 5-8% fat, 4.6-5.2% lactose, 0.7-0.75% ash, 0.5-5.2% lactic acid, 0.12-0.14% Ca and 0.09-0.11% P (Laxminarayana et al. 1952b).

2.4.2. Yoghurt

According to some sources, yoghurt originated in Asia (Oberman 1985). To produce yoghurt, milk from cow, goat, sheep, buffalo or camel is heated to 88-95°C to pasteurize, homogenized and cooked to 42-47°C, before addition of 2-5% lactic starter culture. It is then fermented for 3-6 h until desired acidity obtained and setting yoghurt into soft gel (Campbell-Platt 1987).

The essential microflora in yoghurt consists of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*.

A proportion of 1:1 of *Lactobacillus bulgaricus* and *Streptococcus thermophilus* is considered to be optimum for flavour and texture production (Vedamuthu 1982), but 1:5, 1:10 or 2.1:1.2 are also favourable (Rašić and Kurmann 1978).

The natural yoghurt (without addition) contains 85-90% moisture, and per 100 g dry matter: 30-35 g protein, 7-15 g fat, 43-48 g carbohydrate, 6-8 g ash, 1.6-1.8 MJ (380-420 kcal) energy, 1700 mg K, 1400 mg Ca, 120 mg Mg, 1000 mg P, 0.6 mg Fe, 0.3 mg Cu, 4 mg Zn, 1300 mg Cl, 0.35 mg thiamine, 1.8 mg riboflavin, 1 mg niacin, 7 mg potential niacin from tryptophan, 7 µg free folic acid, 14 µg total folic acid, 0.3 mg vitamin B₆, trace vitamin B₁₂, 60 µg retinol, 35 µg carotene, trace vitamin D, 3 mg vitamin C and

0.2 mg vitamin E (Campbell-Platt 1987).

2.4.3. Chu-ra

Chu-ra, a fermented milk product, is traditionally consumed in Tibet, Nepal and northeast India. During its preparation, yak's milk is heated, curd separated by filtration through a cloth, moulded into rectangular (20-40 cm x 15 cm x 15 cm) loaves and left to ferment at low temperature for several days. The loaves are sliced, and the slices are strung on yak hair twine, and allowed to sun dry (Batra and Millner 1976).

2.5. STARTER CULTURES

2.5.1. Murcha

Murcha, a starter culture, has erroneously been referred to as a rice beer as well (Ray 1906; Batra and Millner 1974, 1976; Hesseltine *et al.* 1988). Murcha is a small rice starch cake, about 4-6 cm in diameter and available in the central and eastern Himalayas. In addition to rice starch, the cakes may also contain berries, roots and leaves of wild native plants (Ray 1906).

Hutchinson and Ram-Ayyar (1925) reported the presence of several efficient saccharifying fungi, namely *Aspergillus oryzae*, *Endomycopsis burtonii*, *Mucor javanicus*, *M. prainii* and *Rhizopus cambodja* in murcha. Batra and Millner (1974) isolated *R. arrhizus*, *M. fragilis*, *M. rouxianus*, and the yeast *Hansenula anomala* var. *schneggii* from murcha. Batra (1981) reported the presence of *E. fibuligera* and *Amylomyces rouxii* in addition to those mentioned earlier (Batra and Millner 1974).

The presence of *Mucor* and *Rhizopus* in murcha samples of Nepal was confirmed by Hesseltine (1983b) and Hesseltine *et al.* (1985, 1988). However, *Amylomyces* was consistently absent in those studies, because of the prevailing low temperature in Nepal. Bacterial count of 2×10^8 /g, yeast count of 6×10^8 /g and mould count of 2.8×10^8 /g were found in murcha samples of Nepal (Hesseltine *et al.* 1988). The lactic acid bacteria in murcha samples of Nepal included mostly *Pediococcus pentosaceus* and few *Streptococcus faecalis* (Hesseltine and Ray 1988). *Saccharomycopsis fibuligera* represents the dominant starch-degrading yeast in murcha, associated with less predominance of *Saccharomyces* and *Pichia* (Hesseltine and Kurtzman 1990).

2.5.2. Ragi, Chinese yeast, bubod and loogpang

The starter preparations go under a variety of names, such as ragi in Indonesia, Chinese yeast or chiu-chu in China and Taiwan, bubod in Philippines and loogpang in Thailand (Hesseltine *et al.* 1988). Except for the Thai loogpang in which the organisms are grown on bran, the predominant forms of the ragi type starters are small (3-6 cm), round and flattened cakes of rice flour (Djien 1977; Pichyangkura and Kulprecha 1977). The use of Chinese yeast was described as early as 531 A.D. in China (Yamazaki 1932).

The starter cultures are made by mixing rice flour with various spices such as ground garlic, black pepper, ginger etc. Some wild herbs are also blended. Water is added to make a thick paste which is kneaded into small flattened cakes. Powdered old starter cultures are sprinkled over the cakes, placed on bamboo tray, fermented at 25-30°C for 2-5 days, and then sun-dried

(Macfadyen 1903; Saono *et al.* 1974, 1982; Tanimura *et al.* 1977; Yeoh 1977; Djien 1977, 1986).

Spices such as garlic, lengkuas, ginger and kapulaga added to ragi may inhibit development of undesirable microorganisms (Soedarsono 1972).

Ragi is used to make tapé, a fermented food of Indonesia. Tapé is a syrup-like product made from glutinous rice (Hesseltine *et al.* 1988). Chinese yeast is used to make Chinese dessert lao-chao (Hesseltine 1983b) and Shaohsingchui, an alcoholic Chinese beverage (Yamazaki 1918). Bubod is used to make basi (sugarcane wine) and binobodan (rice wine) in Philippines (Del Rosario 1980). Loogpang is used to make tapé-like products and rice wine preparation in Thailand (Pichyangkura and Kulprecha 1977).

Ishimaru and Nakano (1969) found *Streptococcus faecalis* and *Lactobacillus plantarum* in ragi and obtained bacterial count as high as 10^{10} in 24 h-old culture. Djien (1972) found the presence of *Amylomyces rouxii*, *Endomycopsis chodati*, *Mucor rouxii* and *Rhizopus* sp. in ragi of Indonesia, and concluded that combination of *Amylomyces* and *Endomycopsis* resulted in good tapé fermentation; while others being unimportant. Toyota and Kozaki (1978) studied the bacteria in ragi and identified them as *Pediococcus pentosaceus*. Hadisepoetro *et al.* (1979) reported the presence of a mould *Zygorhynchus*, two yeasts including *Candida* and *Torulopsis* and a lactic acid bacterium, *Pediococcus* in ragi. The counts of mould, yeasts and bacteria were $3.2-4.0 \times 10^4$, $5.6-14 \times 10^6$ and $3.0-18 \times 10^4$, respectively. The presence of *Pediococcus pentosaceus* and *Streptococcus faecalis* was confirmed by Hesseltine and Ray (1988).

Saono and Basuki (1978, 1979) found that the yeasts isolated from ragi had no proteolytic activities, whereas the mould isolates had amylolytic activities.

Hesseltine et al. (1988) examined viability of *Amylomyces rouxii*, *Mucor* and *Rhizopus* in ragi. While *Amylomyces rouxii* could survive remarkably well when kept at room temperature in a dry state, there was considerable reduction in number of *Mucor* and *Rhizopus* with long period of storage.

Suprianto et al. (1989) reported the active microorganisms to be *Rhizopus* sp., *Saccharomycopsis* sp. and *Streptococcus* sp. in tapé fermentation. The presence of *Saccharomycopsis fibuligera* and *Saccharomycopsis malanga* was reported in ragi (Hesseltine and Kurtzman 1990).

According to Hesseltine et al. (1988), four genera of moulds including *Mucor*, *Amylomyces*, *Chlamydomucor* and *Rhizopus* are involved in Chinese yeast. Hesseltine and Kurtzman (1990) reported the presence of *Saccharomycopsis fibuligera* and *S. malanga* in Chinese yeast with less numbers of *Saccharomyces*, *Pichia* and *Candida*.

Bubod contains *Amylomyces*, *Mucor*, *Rhizopus* and *Saccharomyces* (Tanimura et al. 1977). The microbial count in bubod ranged from 2.1×10^3 - 2.3×10^7 for moulds, 7.4×10^4 - 3.0×10^7 for yeasts and 2.9×10^5 - 4.7×10^7 for lactic acid bacteria (Del Rosario 1980). Hesseltine and Kurtzman (1990) reported the presence of *Saccharomycopsis fibuligera* in bubod.

Loogpang from Thailand contains *Amylomyces*, *Rhizopus*, *Aspergillus*, *Mucor* and *Absidia* (Pichyangkura and Kulprecha 1977).

2.6. FERMENTED BEVERAGES

2.6.1. Millet beverage

In old literatures (Hooker 1854; Riskey 1894; Gorer 1938), there are mentions of fermented millet or marwa beverage of Darjeeling hills and Sikkim. The beverage is also known as 'chang' by the Sikkimese (Riskey 1894) and 'chi' by the Lepchas (Gorer 1938). 'Thumba', the fermented beverage common in Darjeeling, Sikkim and Nepal has been reported by Hesseltine (1965, 1979) and Batra and Millner (1976). *Endomycopsis fibuliger* has been found in thumba of West Bengal (Hesseltine 1979).

2.6.2. Rice beverage

Rice beer or pachwai or murcha or bakhar is probably the most widely consumed fermented beverage in rural Asia. It is prepared by mixing a starter culture with the cooled rice gruel and fermented for 24 h or longer. The beer is decanted and the residue is used as a meal. the fermentation is carried out by several mucoraceous species or *Aspergillus oryzae* that convert starch to sugars, which are fermented by *Hansenula anomala*, *Endomycopsis fibuligera* and *Amylomyces rouxii*. The ethanol content is 3% and the odour of ethylacetate is discernible (Batra and Millner 1976; Batra 1981, 1986).

2.6.3. Barley beverage

Chiang or lugri, a barley-based fermented beverage, is a mild alcoholic, thick, translucent, foamy drink with a sweet-sour taste and somewhat aromatic flavour. It is consumed without

additional carbonation and is usually neither aged nor filtered. The chiang of south-western Tibet, along the Nepal border is sour and aged during storage for one month. Chiang is consumed in this area with addition of 2-3 g of yak butter which floats on the top of beverage (Batra and Millner 1976; Batra 1986).

In the traditional method of its preparation, during late March through May high quality grains from previous year is soaked overnight, dewatered, spread in gunny sacks, incubated for 2-5 days in a warm place and allowed to dry gradually. The grain is further air-dried in the sun, then coarsely ground and mashed. The mash is boiled, cooled, mixed with unmalted crushed grain and fermented for 3-6 days in a cool place. The starter inoculum comes either from the unmalted grain and flowers of diverse plants that are added, or from the portion of beer added to the mash from a previous batch. *Saccharomyces cerevisiae* and *S. uvarum* are found to occur in chiang or lugri (Batra 1986).

3. MATERIALS AND METHODS

3.1. CULTURE MEDIA USED

Anaerobic agar (Claus and Berkeley 1986)

Trypticase	20.0 g
Glucose	10.0 g
NaCl	5.0 g
Agar	15.0 g
Sodium thioglycolate	2.0 g
Sodium formaldehyde sulfoxylate	1.0 g
Distilled water	1000 ml
pH	7.2

APT agar (HiMedia M226)

Arginine hydrolysis test medium (Thornley 1960)

Peptone	1.0 g
Arginine	10.0 g
NaCl	5.0 g
$K_2HPO_4 \cdot 3H_2O$	0.3 g
Phenol red	0.01 g
Agar	4.0 g
Distilled water	1000 ml
pH	7.2-7.4

Basal medium for acid and gas production from carbohydrates

(Gordon *et al.* 1973)

Diammonium hydrogen phosphate	1.0 g
KCl	0.2 g

MgSO ₄ ·7H ₂ O	0.2 g
Yeast extract	0.2 g
Bromocresol purple	0.4 g
Distilled water	1000 ml
pH	7.0

Columbia blood agar base and defibrinated sheep blood

(Oxoid CM331 and SR51)

Davis and Mingioli's broth (Davis and Mingioli 1950)

K ₂ HPO ₄	7.0 g
KH ₂ PO ₄	3.0 g
Sodium citrate·3H ₂ O	0.5 g
MgSO ₄ ·7H ₂ O	0.1 g
(NH ₄) ₂ SO ₄	1.0 g
Glucose (Sterilized separately)	10.0 g
Distilled water	1000 ml
pH	7.0

Esculin hydrolysis test medium

Esculin	1.0 g
Ferric chloride	0.5 g
Peptone	5.0 g
Yeast extract	1.0 g
Agar	20.0 g
Distilled water	1000 ml

Gelatin agar (Sneath and Collins 1974)

Beef extract	3.0 g
Peptone	5.0 g
Agar	20.0 g
Gelatin	10.0 g
Distilled water	1000 ml
pH	7.2

Glucose-yeast extract-peptone (GYE)-CaCO₃ agar(Okada *et al.* 1986)

Glucose	10.0 g
Yeast extract	5.0 g
Peptone	5.0 g
Tween 80	0.5 g
Salt solution	5.0 ml
CaCO ₃ (Sterilized separately in an oven at 180°C for 6 h)	5.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	6.8

Salt solution

MgSO ₄ .7H ₂ O	4.0 g
MnSO ₄ .4H ₂ O	0.2 g
FeSO ₄ .7H ₂ O	0.2 g
NaCl	0.2 g
Distilled water	100 ml

Glucose-yeast extract-peptone water (Kreger-van Rij 1984)

Glucose	20.0 g
Peptone	10.0 g
Yeast extract	5.0 g
Distilled water	1000 ml
pH unadjusted	

Agar (2% w/v) was added to prepare glucose-yeast extract-peptone agar.

Gorodkova agar (Kreger-van Rij 1984)

Glucose	1.0 g
Peptone	10.0 g
NaCl	5.0 g
Agar	20.0 g
Tap water	1000 ml
pH unadjusted	

Malt extract (Lodder and Kreger-van Rij 1952)

Malt extract powder	150.0 g
Demineralized water	1000 ml
pH 5.4	

Malt extract agar (Kreger-van Rij 1984)

Malt extract powder	100.0 g
Agar	20.0 g
Demineralized water	1000 ml
pH 5.4	

Milk agar (Gordon et al. 1973)

Skim milk powder	5.0 g in 50 ml distilled water
Agar	1.0 g in 50 ml distilled water

Autoclaved separately at 121°C for 20 min, cooled to 45°C, mixed together and poured into Petri dishes. The plates were allowed to stand at 37°C for 24 h to dry the surface of the agar.

MRS broth (de Man et al. 1960)

Peptone	10.0 g
Beef extract	10.0 g
Yeast extract	5.0 g
K_2HPO_4	2.0 g
Diammonium citrate	2.0 g
Glucose	20.0 g
Tween 80	1.0 g
Na acetate	5.0 g
$MgSO_4 \cdot 7H_2O$	0.58 g
$MnSO_4 \cdot 4H_2O$	0.28 g
Distilled water	1000 ml
pH	6.2-6.4

Agar (2% w/v) was added to prepare MRS agar.

Nitrate broth (Gordon et al. 1973)

Peptone	5.0 g
Beef extract	3.0 g
KNO ₃	1.0 g
Distilled water	1000 ml
pH	7.0

Nutrient broth (Gordon et al. 1973)

Beef extract	3.0 g
Peptone	5.0 g
Distilled water	1000 ml
pH	6.8

Agar (2% w/v) was added to prepare nutrient agar.

Potato dextrose agar (PDA) (APHA 1967)

Potatoes, peeled and sliced	200.0 g
D-glucose	20.0 g
Agar	20.0 g
Distilled water	1000 ml

Potatoes (200 g) were peeled, sliced and boiled for one h in 1000 ml distilled water. The solution was filtered. The filtrate was added with glucose and agar, and boiled again to dissolve the agar. The volume was made up to 1000 ml.

Sugar basal broth (for lactic acid bacteria) (Garvie 1984)

Peptone	10.0 g
Yeast extract	2.5 g
Tween 80	0.1 g
Bromocresol purple (1.6% w/v in ethanol)	1.0 ml
Distilled water	1000 ml
pH 6.8	

Tributylin agar (Stolp and Gadkari 1981)

Peptone	5.0 g
Yeast extract	3.0 g
Tributylin	10.0 g
Agar	12.0 g
Distilled water	1000 ml
pH 7.4-7.6	

Tryptone-glucose-yeast extract (TGYE) broth

(Mukherjee *et al.* 1965)

Tryptone	5.0 g
Glucose	15.0 g
Yeast extract	2.5 g
Tween 80	1.0 ml
Salt A	5.0 ml
Salt B	5.0 ml
Distilled water	1000 ml

Salt A

K_2HPO_4	100.0 g
KH_2PO_4	100.0 g
Distilled water	1000 ml

Salt B

$MgSO_4 \cdot 7H_2O$	40.0 g
NaCl	2.0 g
$FeSO_4 \cdot 4H_2O$	2.0 g
$MnSO_4 \cdot 7H_2O$	2.0 g
Distilled water	1000 ml

Urea medium (Christensen 1946)Part A

Peptone	1.0 g
Glucose	1.0 g
NaCl	5.0 g
KH_2PO_4	2.0 g
Phenol red	0.012 g
Agar	20.0 g
Distilled water	1000 ml
pH	6.8

Part B

Urea	40.0 g
Distilled water	1000 ml

(Sterilized by filtration)

After sterilization of Part A in test tubes (5 ml/tube), 0.25 ml of sterile Part B was added and made slants.

Voges-Proskauer (VP) broth (Gordon et al. 1973)

Peptone	7.0 g
Glucose	5.0 g
NaCl	5.0 g
Distilled water	1000 ml
pH	6.5

Yeast extract-malt extract (YM) agar (Wickerham 1951)

Yeast extract	3.0 g
Malt extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	20.0 g
Distilled water	1000 ml
pH	5.0-6.0

Yeast nitrogen base (Difco 0392-15-9)

All the media mentioned above were sterilized by autoclaving at 121°C for 15 min, unless mentioned otherwise.

3.2. REAGENTS USED

Burke's iodine solution (Bartholomew 1962)

Iodine	1.0 g
KI	2.0 g
Distilled water	100 ml

Crystal violet stain (Bartholomew 1962)

Crystal violet	2.0 g
95% Ethanol	20 ml
Ammonium oxalate	80 ml

(1% w/v aqueous solution)

Ehrlich-Böhme reagent (Iswaran 1980)

p-Dimethylaminobenzaldehyde	1.0 g
95% Ethanol	95 ml
HCl concentrated	20 ml

Physiological saline (Karki et al. 1983d)

NaCl	8.5 g
Distilled water	1000 ml

Reagent for nitrate reduction test (Norris et al. 1981)

Solution A

Sulphanilic acid	0.8 g
5 N Acetic acid	100 ml

(Glacial acetic acid : water, 1 : 2.5)

Solution B

α -Naphthylamine	0.5 g
5 N Acetic acid	100 ml

The solutions A and B were mixed in equal quantities just before use.

Safranin stain (Norris *et al.* 1981)

2.5% w/v Safranin in 95% ethanol	10 ml
Distilled water	100 ml

All the chemicals used were of the highest purity grade.

3.3. EXPERIMENTAL**3.3.1. Survey on traditional fermented foods**

A survey was conducted in different places of Darjeeling hills and Sikkim to get detailed information on the types, traditional methods of preparation, modes of consumption, ethnic value and distribution of various fermented foods used by the local people. A format (Table 3) was specially prepared for the survey.

3.3.2. Collection of sample

Samples of kinema, sinki, mesu and murcha were collected aseptically in separate sterile glass bottles from different periodic markets, locally called 'hats', of Darjeeling, Kalimpong, Gangtok and Rongli.

Table 3. Format of survey on consumption of fermented foods in Darjeeling hills and Sikkim

District :
 Tehsil/town :
 Village :
 Approximate population :
 Name of informant :
 Date of survey :

Sl No.	Fermented products	Consumption (Yes/No)	Daily/Weekly/ Monthly/ Occasional	Household preparation/ Market purchase	Raw materials: Home cultivation/ Cash purchase
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The bottles of kinema and mesu were kept in an ice-box and transported immediately to the laboratory for analyses. The raw ingredients used for the preparations of kinema, sinki and mesu were also collected for analyses.

3.3.3. Biochemical analysis

3.3.3.1. Moisture

A well-mixed sample (ca 2 g) was accurately weighed into a cooled and weighed Petri dish (provided with cover), previously heated to $130\pm 1^\circ\text{C}$. The sample was uncovered and allowed to dry for 1 h at $130\pm 1^\circ\text{C}$ in a hot air oven. The dish was covered while still in oven, transferred to a dessicator, and weighed soon after reaching room temperature. The process of drying, cooling and weighing was repeated until the two successive weights reached a constant value. Moisture content was calculated by subtracting the final weight from the initial weight (AOAC 1990).

3.3.3.2. Ash

A well-mixed sample (ca 2 g) was accurately weighed into a previously dried and weighed porcelain crucible and placed in a muffle furnace preheated to 600°C . The sample was held at that temperature for 2 h. The crucible was transferred directly to a dessicator, allowed to cool to room temperature and weighed immediately (AOAC 1990). The process of heating for 30 min, cooling and weighing was repeated until the difference between two successive weighings was ≤ 1 mg. The lowest mass was recorded (IS: 5162 1980).

3.3.3.3. pH

A sample (100 g) was mixed with 20-50 ml carbon dioxide-free distilled water in a waring blender (Bajaj, India) for 1 min. The temperature of the paste prepared was equilibrated to 25°C and the pH was noted (AOAC 1990) using a pH meter (Systronics type 335).

3.3.3.4. Titratable acidity

A well-mixed sample (10 g) was blended with 90 ml carbon dioxide-free distilled water for 1 min. The mixture was filtered, and 25 ml of the filtrate was titrated with 0.1 N NaOH to an end point of phenolphthalein (0.1% w/v in 95% ethanol) (AOAC 1990).

$$\% \text{ Titratable acid content (as lactic acid)} = \frac{100 \times \text{ml of NaOH} \times \text{N of NaOH} \times 0.09}{\text{Weight of sample (g)}}$$

3.3.3.5. Total nitrogen

The method as described in AOAC (1990) was followed. Approximately 1 g accurately weighed sample, taken in a digestion flask, was added with 0.7 g HgO, 15 g powdered K₂SO₄ and 25 ml concentrated H₂SO₄. The flask was heated gently until frothing ceased, boiled briskly until the solution became clear, and then continued the boiling for about 1 h. The solution was transferred quantitatively to a round bottom flask, and mixed with approximately 100 ml distilled water and 25 ml 4% w/v aqueous Na₂S to precipitate mercury. A pinch of zinc granules to prevent bumping and a layer of 40% w/v aqueous NaOH were added carefully. The flask was immediately connected to a distillation apparatus and the tip of the condenser was immersed in standard (0.1-0.5 N) H₂SO₄ containing

about 5 drops of methyl red indicator (0.5% w/v methyl red in ethanol). The flask was rotated to mix the contents thoroughly and heated until all the ammonia had distilled. The receiver was removed and the tip of the condenser was washed with distilled water. The remaining acid in the receiver was titrated with standard (0.1-0.5 N) NaOH solution. The blank determination on reagents was considered for correction.

$$\% N = [(ml \text{ of standard acid} \times N \text{ of standard acid}) - (ml \text{ of standard NaOH} \times N \text{ of standard NaOH})] \times 1.4007 / \text{Weight of sample (g)}$$

3.3.3.6. Protein and non-protein nitrogen

A sample (0.5 g) was mixed with 30 ml 10% w/v cold trichloroacetic acid and allowed to stand at 5°C for 20 h. The solution was centrifuged at 8500 g for 10 min. Nitrogen contents in the supernatant fraction (non-protein nitrogen) and the precipitate (protein nitrogen) (Nirenberg and Matthaei 1961) were measured by micro-Kjeldahl method as described in Section 3.3.3.5.

3.3.3.7. Soluble nitrogen

A well-mixed sample (ca 2 g) was shaken with 100 ml distilled water for 45 min on a rotary shaker (120 rpm) and centrifuged at 8000 g for 10 min. The supernatant was filtered through Whatman No. 2 filter paper (Shieh et al. 1982) and the nitrogen in the known volume of the filtrate was determined using the micro-Kjeldahl procedure as described in Section 3.3.3.5.

3.3.3.8. Protein

Protein contents in the sample were determined by multiplying total nitrogen value with 5.7 for the soya bean product and 6.25 for the others (AOAC 1990).

3.3.3.9. Fat (crude)

The method as described in IS: 1167 (1965) was followed.

Approximately 5 g accurately weighed sample was carefully placed in about 20 ml concentrated HCl containing in a beaker and boiled for 10 min. After cooling to room temperature, the content was quantitatively transferred to a Rose-Gottlieb fat extraction tube using 25 ml diethyl ether. The tube was stoppered with a bark cork and shaken vigorously for 1 min. The beaker was washed with 25 ml petroleum ether and the wash liquid was poured into the tube.

After vigorous shaking, the tube was allowed to stand until the upper liquid was clear. The ether solution was decanted into a preweighed Erlenmeyer flask. The tube was washed with a mixture of equal parts of both the solvents, and the washings were added to the flask. Extraction of the liquid remaining in the tube was repeated twice, using 25 ml petroleum ether. The solvent was evaporated completely on a steam bath. The fat was dried in an oven at 100°C to constant weight.

3.3.3.10. Free fatty acidity

An accurately weighed (ca 5 g) sample was dissolved in 25 ml 95% ethyl alcohol neutralized previously by 0.1 N NaOH, using phenolphthalein (0.01% w/v in 95% ethanol). After mixing, the

solution was heated to boiling on water bath. The mixture was titrated with 0.1 N NaOH until a faint pink colour persisted (Shieh et al. 1982).

$$\% \text{ Fatty acid content (as linoleic acid)} = \frac{100 \times \text{ml of NaOH} \times \text{N of NaOH} \times 0.192}{\text{Weight of sample (g)}}$$

3.3.3.11. Carbohydrate

The carbohydrate content was calculated by difference (Standal 1963).

$$100 - (\% \text{ protein} + \% \text{ fat} + \% \text{ ash})$$

3.3.3.12. Energy value

The energy value of a sample was determined by multiplying its percent protein, fat and carbohydrate contents by the factors 4, 9 and 4, respectively, and adding all the multiplication values to get kcal (4184 kcal = 1 Joule) per 100 g.

3.3.4. Microbial analysis

3.3.4.1. Isolation and maintenance

A sample (10 g) was mixed with 90 ml sterile physiological saline containing in an Erlenmeyer flask by placing it on a rotary shaker (120 rpm) for 1 h. Decimal dilution series was prepared in physiological saline. One ml of appropriately diluted suspension of sample was mixed well with molten (45°C) medium and poured into plates. Nutrient agar plates were incubated at 37°C for 18- 24 h; MRS, APT and GYP-CaCO₃ agar plates were incubated at 30°C in a spontaneously extinguished candle jar for 48-72 h; and YM agar and

PDA (supplemented with 10 IU/ml benzylpenicillin and 12 $\mu\text{g/ml}$ streptomycin sulphate; Batra and Millner 1974) plates were incubated at 28°C for 48 h. Usually, the incubated plates containing 50-300 colonies were selected for enumeration. The colonies appeared were counted as colony forming units (cfu) per g sample. The isolated colonies of bacteria, yeasts and moulds were checked for purity and picked up on slants of their respective media on which they developed during isolation. The slant cultures were maintained at 4°C by subculturing after every two months. All the representative strains of the different groups of the isolates were deposited in the Culture Collection of the Microbiology Laboratory of the Department of Botany, University of North Bengal.

3.3.4.2. Taxonomic studies on bacterial isolates

3.3.4.2.1. Gram staining

The method of Bartholomew (1962) was followed. A suspension of a 24 h-old bacterial culture on slant was prepared in distilled water. A drop of that suspension was taken on a grease-free slide and a smear was made. It was then heat-fixed, flooded by crystal violet stain for 1 min, and washed for 5 s with water. The smear was flooded with Burke's iodine solution, allowed to react for 1 min, and washed again for 5 s with water. Holding the slide against a white surface, 95% ethanol was poured dropwise from the top edge of the slide until no more colour came out from the lower edge of the slide. After washing with water, the smear was stained with safranin for 1 min and washed again with water. The slide was air-dried and observed under oil-immersion objective.

3.3.4.2.2. General morphology

An air-dried (not heat-fixed) smear of a 24 h-old bacterial culture was stained for 30 s with safranine, washed in water, air-dried (Norris *et al.* 1981) and observed under oil-immersion objective. Cell dimension was measured with a standardized ocular micrometer.

3.3.4.2.3. Motility

A drop of a 24 h-old culture in nutrient or MRS broth was used to prepare a hanging drop in a cavity slide. The drop was observed using a phase contrast microscope (Olympus BH2-PC-PA-1).

3.3.4.2.4. Production of catalase

A 24 h-old slant culture was flooded with 0.5 ml of 10% hydrogen peroxide solution and observed for the production of gas bubbles, indicating the presence of catalase (Norris *et al.* 1981).

3.3.4.2.5. Growth in sodium chloride

Nutrient broth (3 ml/tube) containing 5 and 7% w/v sodium chloride were inoculated with a loopful of culture, and incubated for 3 days at 37°C in a slanting position to improve aeration (Norris *et al.* 1981). For enterococci and lactic acid bacteria, tubes of TGYE broth (5 ml/tube) containing 4, 6.5 and 18% w/v sodium chloride were inoculated with a loopful of culture and incubated at 30°C for 7 days.

3.3.4.2.6. Acid and gas from carbohydrates

Tubes of 10 ml basal medium (for acid and gas production from carbohydrates) containing 0.5% w/v of different carbohydrates and

inverted Durham tubes were inoculated with the isolates and incubated at 37°C for 3 days (Gordon *et al.* 1973). For enterococci and lactic acid bacteria, the method was based on Pederson and Albury (1950) and Okada *et al.* (1986). Tubes of 10 ml sugar basal broth containing 2% w/v sugar and inverted Durham tubes were inoculated and incubated at 30°C for 10 days. Accumulation of gas in the inverts indicated positive result.

3.3.4.2.7. Production of indole

Cells were grown at 37°C (30° for enterococci and lactic acid bacteria) in 10 ml Davis and Mingioli's broth, prepared by replacing ammonium sulphate with L-tryptophan (0.1% w/v) and supplementing with yeast extract (0.02% w/v). Ehrlich-Böhme reagent (1-2 ml) was layered on 3,5 and 7 days-old broth culture. Formation of a red ring at the culture-reagent interface was considered as an indication of positive result (Iswaran 1980).

3.3.4.2.8. Voges-Proskauer reaction

Tubes of 10 ml Voges-Proskauer broth were inoculated with the isolates and incubated at 37°C for 3 days (30°C for 7 days for enterococci and lactic acid bacteria). To the culture, 0.6 ml 5% w/v ethanolic α -naphthol and 0.2 ml 40% w/v aqueous potassium hydroxide were added and kept for 1 h at room temperature for the production of a pink colour, indicating the positive reaction. Initial and final pH of the broth were measured using the pH meter.

3.3.4.2.9. Hydrolysis of gelatin

Gelatin agar plates were streaked with the isolates and incubated

at 37°C for 24 h. For enterococci and lactic acid bacteria, the streaked MRS agar plates containing 1% w/v gelatin were incubated at 30°C for 3 and 5 days. Plates were then flooded with 10 ml 1 N sulphuric acid saturated with ammonium sulphate. Hydrolysis was indicated by a clear zone, under and around the growth, in contrast to the opaque precipitate of unchanged gelatin (Sneath and Collins 1974).

3.3.4.2.10. Hydrolysis of arginine

Tubes of 5 ml arginine hydrolysis test medium were inoculated by stabbing. Immediately after inoculation, a layer (ca 1 cm) of sterile mineral oil was added over the stab. The tubes were incubated at 30°C for 3 days and observed for the change in colour from yellow to red, indicating the formation of ammonia from arginine (Lelliott *et al.* 1966).

3.3.4.2.11. Reduction of nitrate

Cultures were grown in 5 ml nitrate broth incubated at 30°C. After 3, 7 and 14 days, 1 ml of the culture was mixed with 3 drops of the reagent for nitrate reduction test and observed for the development of a red or yellow colour, indicating the presence of nitrite. A small amount of zinc dust was added to the tube that was negative even after 14 days and observed for the development of red colour, indicating the presence of nitrate *i.e.*, absence of reduction (Norris *et al.* 1981).

An alternative method was also followed. A strip of filter paper moistened with 10% w/v aqueous potassium iodide and then with a few drops of 1 N hydrochloric acid was touched with a drop

of the culture. It was observed for the production of purple colour, indicating the presence of nitrite (Claus and Berkeley 1986).

3.3.4.2.12. Hydrolysis of fat

Surface-dried plates of tributyrin agar were streaked with the isolates and incubated at 37°C for 24 h (30°C and 4 days for enterococci and lactic acid bacteria). Lipolytic activity was indicated by the formation of clear zones around the streaks (Stolp and Gadkari 1981).

3.3.4.2.13. Anaerobic growth

Anaerobic agar medium was distributed into culture tubes in amount sufficient to give 7.5 cm depth of the medium and sterilized by autoclaving at 121°C for 20 min.

The tubes were inoculated with a small (outside diameter 1.5 mm) loopful of 24 h-old nutrient broth culture by stabbing up to the bottom of the column. They were incubated at 37°C for 3 and 7 days, and observed for growth along the length of the stab (anaerobic) and on the surface of the agar (aerobic) (Claus and Berkeley 1986).

3.3.4.2.14. Decomposition of casein

Milk agar plates were streaked with 24 h-old cultures and examined after incubating at 37°C for 3 days (30°C and 7 days for enterococci and lactic acid bacteria) for any clearing of casein around and underneath the growth (Gordon et al. 1973).

3.3.4.2.15. Hydrolysis of esculin

Slants of esculin hydrolysis test medium were inoculated and incubated at 30°C for 7 days. Esculin hydrolysis was indicated by blackening of the medium (Facklam and Wilkinson 1981).

3.3.4.2.16. Growth at different pH

The pH of MRS broth was adjusted to different levels using 1 N HCl or 10% w/v NaOH. The medium was distributed into tubes containing 10 ml in each. They were autoclaved, cooled to room temperature and inoculated with 0.2 ml of 48 h-old MRS broth culture. The tubes were incubated at 30°C for 24 h and observed for growth (Hesseltine and Ray 1988).

3.3.4.2.17. Growth in 0.1% methylene blue milk

Tubes containing 10 ml 5% w/v milk and 0.1% w/v aqueous methylene blue were sterilized, inoculated and tightly capped. Following incubation at 30°C for 3 days, the tubes were observed for decolouration, indicating the growth of inoculum and the consequent reduction of methylene blue.

3.3.4.2.18. Growth at different temperatures

Nutrient agar slants were prepared to determine ability to grow at 5°C intervals within a range of 10-60°C. The slants were immersed in water bath at appropriate temperatures until equilibrated, and then inoculated. Growth of the culture was observed after 3 days at 55°C or higher, after 5 days at 30-50°C, after 14 days at 20 and 25°C, and after 21 days at 10 and 15°C (Claus and Berkeley

1986). For lactic acid bacteria, tryptone-glucose-yeast extract broth was inoculated and incubated at 15, 30, 37, 45 and 50°C for a maximum period of 21 days. The growth was checked visually.

3.3.4.2.19. Haemolysis

The method was based on Hardie (1986). Sterile and molten Columbia blood agar base was cooled to 50°C, mixed with defibrinated sheep blood and poured into Petri dishes. The plates were streaked with the isolates and incubated under semi-anaerobic condition (candle jar) for 48 h at 37°C.

3.3.4.2.20. Requirement of growth factors

The test was performed by growing the culture through several transfers of it in Davis and Mingioli's broth at 37°C, and monitoring visually.

3.3.4.2.21. API tests

For API tests, the isolates were grown on sterile cellophane (Grade 325P British Cellophane, Avonmouth) overlaid plates to avoid nutrient contamination. The *Bacillus* strains were grown at 37°C for 16 h on nutrient agar and the *Enterococcus* strains were grown on MRS agar. The growth was harvested in 2 ml sterile normal saline which was used to prepare two further suspensions, corresponding to 10^8 cells/ml: (i) for API 50CH strips, in 10 ml of respective API 50CHB (*Bacillus*) or API 50CHS (*Streptococcus*) medium, and (ii) for API 20E strips, in 5 ml sterile distilled water. The incubation box was prepared by distributing about 10

and 5 ml of sterile water into the honey combed base of the 50CH and 20E trays, respectively to create humid chambers. The strips were unpacked and placed them in the trays. In API 50CH tests, only the tubes (not the cupules) were filled with the bacterial suspension. For the *Bacillus* strains the cupules were left blank, whereas for the *Enterococcus* strains they were filled with sterile mineral oil. The inoculated 50CH strips were incubated at 30°C for 48 h and read at 24 and 48 h. During incubation, the strips were kept tilted approximately 5° away from the cupules in order to trap any gas evolved. A test scoring positive at either reading time was considered positive. For the API 20E tests, filling of tubes and cupules were made following the manufacturer's instruction. The box was incubated at 37°C. After 24 h, the strips were read by referring to the manufacturer's interpretation table. All spontaneous reactions were recorded. Tests for VP, TDA, IND, nitrate and N₂ were done by adding the prescribed chemicals or reagents.

3.3.4.3. Taxonomic studies on yeast isolates

3.3.4.3.1. General morphology

Malt extract and glucose-yeast extract-peptone water (30 ml each) containing in separate Erlenmeyer flasks (100 ml) were inoculated with an actively growing (24 h-old) yeast culture. They were incubated at 28°C for 3 days and observed for cellular morphology and mode of vegetative reproduction (Kreger-van Rij 1984). Dimension of cells was measured with a standardized ocular micrometer.

Malt extract agar and glucose-yeast extract-peptone agar were streaked with actively growing (24 h-old) yeast isolates, incubated at 28°C for 3 days and observed for the colony characteristics.

3.3.4.3.2. Formation of pseudomycelium and true mycelium

For slide culture preparation, a Petri dish, containing a U-shaped glass rod supporting two glass slides, was autoclaved at 121°C for 15 min. Molten (45°C) potato dextrose agar was poured onto the slides. The solidified agar on the slides was inoculated very lightly with a yeast in two lines along each slide. A sterile coverslip was placed over part of the lines. Some sterile water was poured into the Petri dish to prevent the agar from drying out. The culture was then incubated at 28°C for 4 days. For observation, the slides were taken out of the Petri dish and the agar was wiped off the back of the slide. The edges of the streak under and around the coverslip were examined microscopically for the formation of pseudomycelium or true mycelium (Kreger-van Rij 1984).

3.3.4.3.3. Characteristics of asci and ascospores

A yeast extract-malt extract agar (presporulation medium) slant was streaked with a 24 h-old culture of yeast and incubated at 28°C for 2 days. This was used as an inoculum for gorodkova agar and potato dextrose agar (sporulation medium) slants. They were incubated at 28°C for 3 days and examined at weekly intervals up to 4 weeks for the observation of asci and ascospores. A heat-fixed smear was covered with 5% w/v aqueous malachite green for 30 s, heated to steaming 3-4 times over the flame of a spirit lamp and counterstained with safranin for 30 s.

3.3.4.3.4. Reduction of nitrate

The method followed was the same as described in Section 3.3.4.2.11, excepting that the temperature of incubation was 28°C.

3.3.4.3.5. Hydrolysis of urea

Christenses's urea agar plates were streaked with 24 h-old yeast, incubated at 28°C for 3 days and observed for any change in colour of the medium.

3.3.4.3.6. Hydrolysis of fat

The method followed was the same as described in Section 3.3.4.2.12, excepting that the plates were incubated at 28°C.

3.3.4.3.7. Growth at 37°C

Malt extract agar slants were inoculated with 24 h-old yeasts, and incubated at 37°C for 2 days (Kreger-van Rij 1964).

3.3.4.3.8. API tests

For API tests, the isolates were grown at 30°C for 24 h on yeast extract-malt extract agar overlaid with cellophane. The growth was harvested in 20 ml yeast nitrogen base. After thorough shaking, the suspension was incubated at 30°C for 4 h and used for inoculating the API 50CH strips. Following incubation at 30°C up to 10 days, the strips were observed for any change in colour in the tubes.

3.3.4.3.9. Fermentation of sugars

The method was based on Kreger-van Rij (1984). Cells were grown at 30°C for 18 h on malt extract agar plates overlaid with cellophane. The growth was harvested in 2.5 ml filter-sterilized yeast nitrogen base. After thorough mixing, the cell suspension was incubated at 30°C for 4 h. Tubes of 10 ml yeast nitrogen base supplemented with 2% w/v filter-sterilized sugars containing Durham tubes were inoculated with the above-mentioned inoculum preparation. The tubes were incubated at 25°C and shaken regularly to observe for any accumulation of gas in the inverts.

3.3.4.4. Taxonomic studies on mould isolates

3.3.4.4.1. General morphology

The moulds were grown on potato dextrose agar plate at 28°C for 2 days. The size of the sporangiospores was measured with a standardized ocular micrometer.

3.3.4.4.2. Hydrolysis of starch

Starch agar (2% w/v starch and 2% w/v agar) plates were streaked with the mould isolates and incubated at 28°C for 3 days. The plates were flooded with Burke's iodine solution and observed for any formation of a clear zone around and underneath the growth, indicating the presence of amylase.

3.3.4.5. Identification of isolates

Bacteria were identified following the taxonomic keys of Starr *et al.* (1981) and Sneath *et al.* (1986). Yeasts were identified

according to the criteria laid down by Barnett *et al.* (1983) and Kreger-van Rij (1984). Moulds were identified according to Zycha and Siepman (1969) and Hesseltine and Ellis (1973).

3.3.5. Optimization of traditional process parameters

Various process parameters involved in the production of fermented foods were considered for optimization. The sensory attributes of the laboratory-made samples were evaluated immediately after sampling by a panel of seven trained judges. The formats of the score cards (Tables 4-6), specially prepared for the purpose, were based on the score card prepared by Patil and Gupta (1986).

3.3.6. Microbial and biochemical changes accompanying fermentation

The selected fermented foods were prepared in the laboratory under optimized conditions and studied for succession of the microorganisms and selected biochemical parameters.

3.3.7. Testing each of the isolates or combinations thereof for producing kinema

Bacillus, *Enterococcus* and yeast inocula were prepared by introducing approximately 5 ml sterile distilled water onto 24 h-old (37°C) nutrient agar, 48 h-old (37°C) MRS agar and 48 h-old (28°C) yeast extract-malt extract agar slant cultures, respectively, isolated previously from the fermenting materials. The tubes were agitated for 30 s with a cyclomixer (Remi, India). The number of cells in the suspension was determined using a Neubauer's counting chamber and a phase contrast microscope. The suspensions were used

Table 4. Format of sensory score card for kinema

Name Date Time

Please rate these samples for quality attributes according to the following grade descriptions and scoring:

Attributes	Defects	Slight	Distinct	Pronounced	Sample No.		
					A	B	C
Flavour (smell), 50	Flat	39	35	33			
Normal range: 38-47	Rotten	35	33	31			
	Raw beany	34	28	26			
Body and texture, 45	Dry	37	34	32			
Normal range: 38-44	Watery	35	30	20			
Colour, 5	Whitish	3	2	1			
Normal range: 4-5							
TOTAL SCORE:							

Grading of kinema:

Total score	Grade
-------------	-------

92-100 Excellent

82- 91 Good

72- 81 Fair

62- 71 Poor

< 61 Bad

.....
(Signature of the judge)

Requirements of high grade kinema:

Flavour: Nutty with ammoniacal odour

Body and texture: Highly sticky or
mucilaginous and slightly pasty

Colour: Brown

Table 5. Format of sensory score card for sinki

Name Date Time

Please rate these samples for quality attributes according to the following grade descriptions and scoring:

Attributes	Defects	Slight	Distinct	Pronounced	Sample No.		
					A	B	C
Taste, 60	Bitter	47	44	38			
Normal range: 50-57							
Flavour (smell), 35	Flat	27	26	22			
Normal range: 28-34	Raw radish	25	23	21			
	Rotten	20	18	15			
Colour, 5	Yellowish	3	2	1			
Normal range: 4-5							
TOTAL SCORE:							

Grading of sinki:

<u>Total score</u>	<u>Grade</u>
92-100	Excellent
82- 91	Good
72- 81	Fair
62- 71	Poor
< 61	Bad

.....
(Signature of the judge)

Requirement of high grade sinki:

Taste : Soury

Flavour: Acidic (typical sinki flavour)

Colour : White, when fresh

Table 6. Format of sensory score card for mesu

Name Date Time

Please rate these samples for quality attributes according to the following grade descriptions and scoring:

Attributes	Defects	Slight	Distinct	Pronounced	Sample No.		
					A	B	C
Taste, 60 Normal range: 50-57	Bitter	47	44	38			
Flavour (smell), 35 Normal range: 28-34	Flat Rotten	27 25	26 23	22 21			
Colour, 5 Normal range: 4-5	Yellowish	3	2	1			
TOTAL SCORE:							

Grading of mesu:

Total score Grade

92-100 Excellent

82- 91 Good

72- 81 Fair

62- 71 Poor

< 61 Bad

.....
(Signature of the judge)

Requirements of high grade mesu:

Taste : Soury

Flavour : Acidic (typical mesu flavour)

Colour : White

as inocula, so that the concentration of *Bacillus* cells and spores, *Enterococcus* cells and yeast cells became 10^5 - 10^6 , 10^4 - 10^5 and 10^2 - 10^3 , respectively per g of soaked and sterilized (121°C for 15 min) beans kept in a 300 ml glass jar with cotton plug. The rationale behind selecting the inoculum load was based on the observation on the initial microbial load in soya beans during kinema production.

The products were evaluated organoleptically using the method as described in Section 3.3.5.

3.3.8. Growth of the *Bacillus* isolate at different oxygen levels

Three sets of nutrient agar plates which had been dried at 37°C for 24 h after preparation were streaked and three sets of nutrient broth in test tubes were inoculated with *Bacillus subtilis* DK-W1. One of the three sets was incubated semi-anaerobically in a candle jar and the third anaerobically with CO₂, H₂ and N₂ in the proportion of 10 : 10 : 80, all at 37°C. Average sizes of the colonies developed under each oxygen tension condition were recorded. Counts of the organisms in the incubated nutrient broth in test tubes were determined after 24 h using pour plate method (Ogbadu and Okagbue 1988).

3.3.9. Pure culture fermentation for kinema production

The proven producing strain (DK-W1) of *Bacillus subtilis* was grown on nutrient agar slant for 24 h. A cell-spore suspension prepared at a concentration of 10^8 - 10^9 /ml was used as an inoculum. Two hundred g of 8 h-soaked soya beans were sterilized at 121°C for 15 min and mixed with 0.5 ml of the inoculum suspension (Sakurai

1960). The inoculated beans were allowed to ferment at 45°C for determining the optimum period of fermentation. The samples were evaluated organoleptically following the method as described in Section 3.3.5.

3.3.10. Microbial and biochemical changes during fermentation of sterilized soya beans inoculated with *Bacillus subtilis* DK-W1

Two hundred g of 8 h-soaked soya beans were sterilized at 121°C for 15 min, and incubated with 24 h-old *Bacillus subtilis* DK-W1 culture at a load of 10^4 - 10^5 cells-spores/g fresh weight. The inoculated beans in a glass jar with cotton plug were allowed to incubate at 45°C till 18 h. Sampling was made at certain intervals for analysis.

3.3.11. Statistical analysis

The data obtained were analyzed statistically by determining analysis of variance using the least square design technique (Snedecor and Cochran 1967).

4. RESULTS

4.1. TRADITIONAL METHODS OF PREPARATION, MODE OF CONSUMPTION AND ETHNIC VALUE OF THE FERMENTED FOODS UNDER SURVEY

Following the survey in Darjeeling hills and Sikkim, a detailed information was obtained on different traditional fermented foods used by the people of these places (Table 7).

4.1.1. Fermented legumes

4.1.1.1. Kinema

Kinema is a soya bean based food traditionally consumed by the non-Brahmin Nepalis. It is now popular among the Lepchas and the Bhutias who call it 'satlyangser' and 'bari', respectively. Two indigenous cultivars, 'local yellow' and 'local brown' (Fig. 2a and b), of soya bean [*Glycine max* (L.) Merrill] are commonly grown in these regions. For kinema preparation, the beans of the 'local yellow' cultivar are commonly used. These beans are small (5-8 mm, average 6 mm) with smooth yellow seed coat and dark brown hilum.

The traditional method of kinema preparation is shown in Fig. 3 and 4. Soya beans are cleaned, washed, soaked in water overnight at ambient temperature (10-25°C) and excess water is drained off. After soaking, the beans increase by more than two times their original weight and become soft. Soaked beans, with fresh water, are cooked by boiling in open cooker upon earthen oven until they can be crushed easily between the finger tips. Excess water is drained off. Boiled seeds are put into a large wooden mortar and crushed lightly by a wooden pestle to dehull most of the seeds and to break the seeds to maximum one-fourth of the intact seeds. A

Table 7. Traditional fermented foods of Darjeeling hills and Sikkim

Food	Substrate	Nature and use
A. Fermented legumes		
Kinema	Soya beans	Cooked beans covered with sticky materials; served as curry with boiled rice
Masayura	Black gram	Ball-like, hollow; used as spicy condiment
B. Fermented vegetables		
Gundruk	Leafy vegetables	Dried, sour vegetable; eaten as soup or pickle
Sinki	Radish tap root	Dried, sour vegetable; eaten as soup or pickle
Mesu	Bamboo shoot	Fresh and soury; eaten as pickle
Khalpi	Cucumber	Fresh and soury; eaten as pickle
C. Fermented cereal preparation		
Shel roti	Rice flour	Fermented batter deep fried, pretzel or ring-shaped; eaten as confectionary bread
D. Fermented dairy products		
Dahi	Cow's milk	Thick gel; acidic, non-alcoholic savory

Food	Substrate	Nature and use
Kachcha churpi	Cow's milk	Soft mass; used as condiment
Churpi	Cow's/yak's milk	Hard mass; eaten as masticatory
Dudh churpi	Cow's/yak's milk	Hard mass; eaten as masticatory
E. Fermented meat		
Sukako masu	Mutton/beef/ pork/yak meat	Smoked meat; eaten as curry
F. Starter culture		
Murcha	Rice flour	Solid, flattened ball
G. Fermented beverages		
Jnard	Finger millet/ rice/starchy material	Mildly sweet, acidic and alcoholic drink
Raksi	Rice/finger millet	Distilled part of jnard

small amount of firewood ash is often added and mixed. The whole soya bean grits are then wrapped with fresh fern (*Athyrium* sp..) or *Leucosceptum canum* Smith leaves. At present, polythene bags are also sometimes used. The wrapped mass is covered with sackcloth, kept in a bamboo basket and placed in a warm place, usually above an earthen oven in kitchen, for 1-2 days during summer or 2-3 days

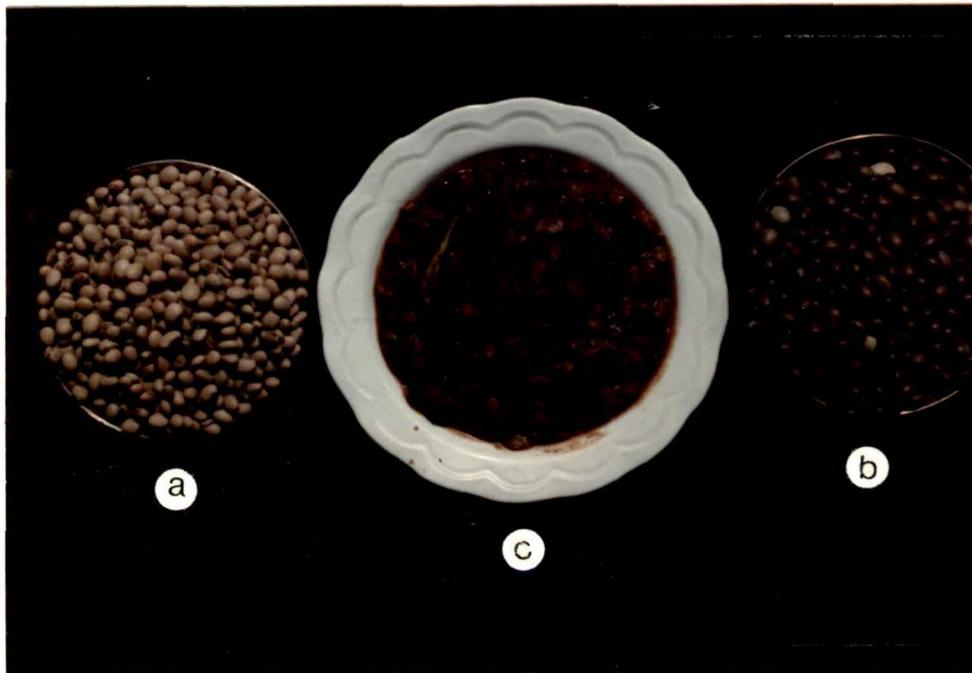


Fig. 2. Seeds of 'local yellow' (a) and 'local brown' (b) cultivars of soya bean [*Glycine max.* (L.) Merrill] and kinema curry (c)



Fig. 4. Kinema preparation in a house of rural Sikkim. (a) Dewatering after cooking; (b) transferring cooked seeds into mortar; (c) crushing seeds with a pestle; (d) arranging for wrapping Contd



Fig. 4 (Contd). (e) transferring soya bean grits on fern leaves in a basket; (f) covering with fern leaves; (g) final covering with sackcloth; and (h) leaving in kitchen for fermentation

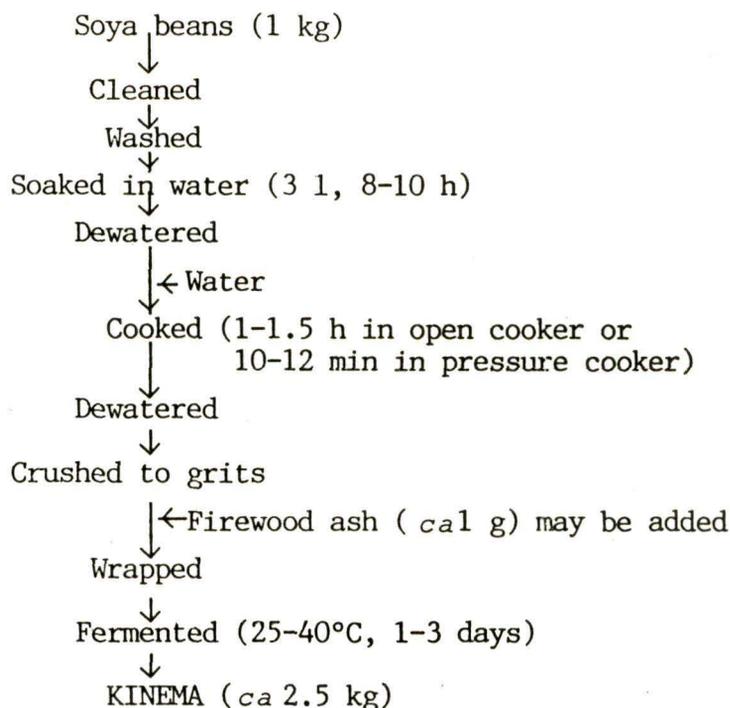


Fig. 3. Flow sheet of kinema preparation

during winter. The desired state of fermentation is indicated by the formation of a typical kinema flavour dominated by ammonia. During fermentation, soya beans become covered with a viscous fluid which appears to determine, to a large extent, the quality of the finished product. Kinema shows long, stringy threads when touched with fingers; longer the thread, better is the quality of kinema. Fresh kinema keeps for 2-3 days during summer and a maximum of one week in winter. The shelf-life is often lengthened to one month or more by drying in the sun or by keeping above an earthen oven in kitchen.

Kinema is used to give a pleasant, nut-like flavour to curry. Kinema is eaten as side dish with rice. Sometimes, it is mixed with vegetables. Fresh kinema (Fig. 5) is fried in edible oil and



Fig. 5. Freshly prepared kinema



Fig. 6. Kinema (a), gundruk (b) and sinki (c) being marketed at Darjeeling. A silver mug for measuring kinema is also seen.

mixed with salt, onion, green chillies and tomato. A little water is added to make a thick curry (Fig. 2c).

Kinema is sold in all periodic local markets of these regions. Usually, it is sold by volume taking in a silver mug (Fig. 6a) and packing in leaves of *Ficus hookeriana* Corner (locally called, 'nevara') loosely tied by straw.

4.1.1.2. Masayura

Masayura is a rare black gram [*Vigna mungo* (L.) Hepper] product, traditionally consumed by certain castes of Nepalis. It is prepared (Fig. 7) during winter only. Black gram seeds are cleaned, washed

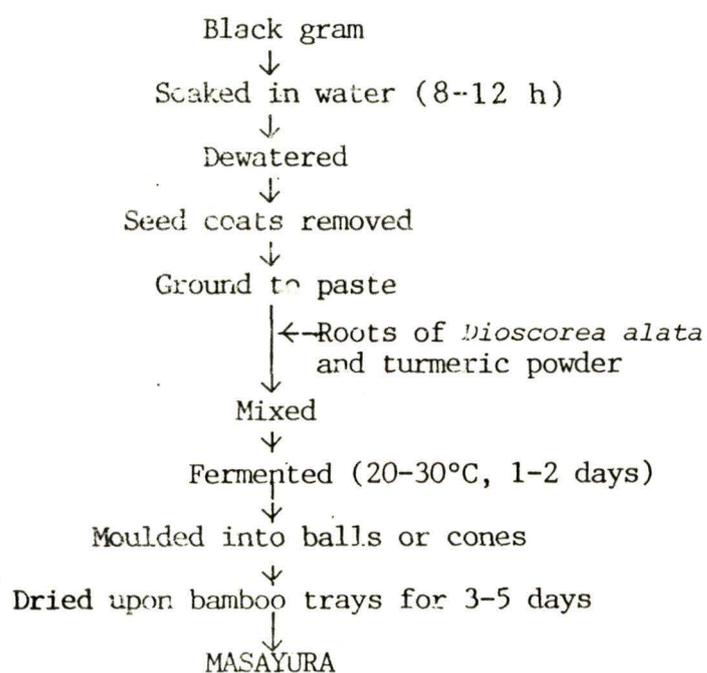


Fig. 7. Flow sheet of masayura preparation

and soaked overnight. Excess water is drained off. Seeds are dehulled by pressing through hands and the hulls are blown off. They are ground to paste which is added with turmeric powder and root of *Dioscorea alata* L., locally called 'ghar tarul', and fermented in kitchen for 1-2 days. The batter is then hand-moulded into balls or cones and sun-dried on bamboo trays.

Shelf-life of masayura is about one year.

Masayura is a hollow, brittle and friable ball (Fig. 8), consumed as a condiment or adjunct in cooking vegetables.



Fig. 8. Masayura, showing cavities in some inverted ones

4.1.2. Fermented vegetables

4.1.2.1. Gundruk

Gundruk, a leafy vegetable product, is traditionally consumed by the Nepalis. Now it is popular among all the ethnic groups of these regions. The Bhutias call it 'lo-kyur'. Gundruk is usually prepared during the months of December to February when the weather is less humid and there is ample supply of green vegetables. The substrate of gundruk is usually the leaves of 'rayo' [*Brassica rapa* L. ssp. *campestris* (L.) Clapham var. *cuneifolia* Roxb.]. Other leaves, such as radish (*Raphanus sativus* L.), cauliflower (*Brassica oleracea* L. var. *botrytis* L.) and 'shimrayo' [*Rorippa nasturtium-aquaticum* (L.) Hayek] are also used.

In the traditional method of gundruk preparation (Fig. 9 and

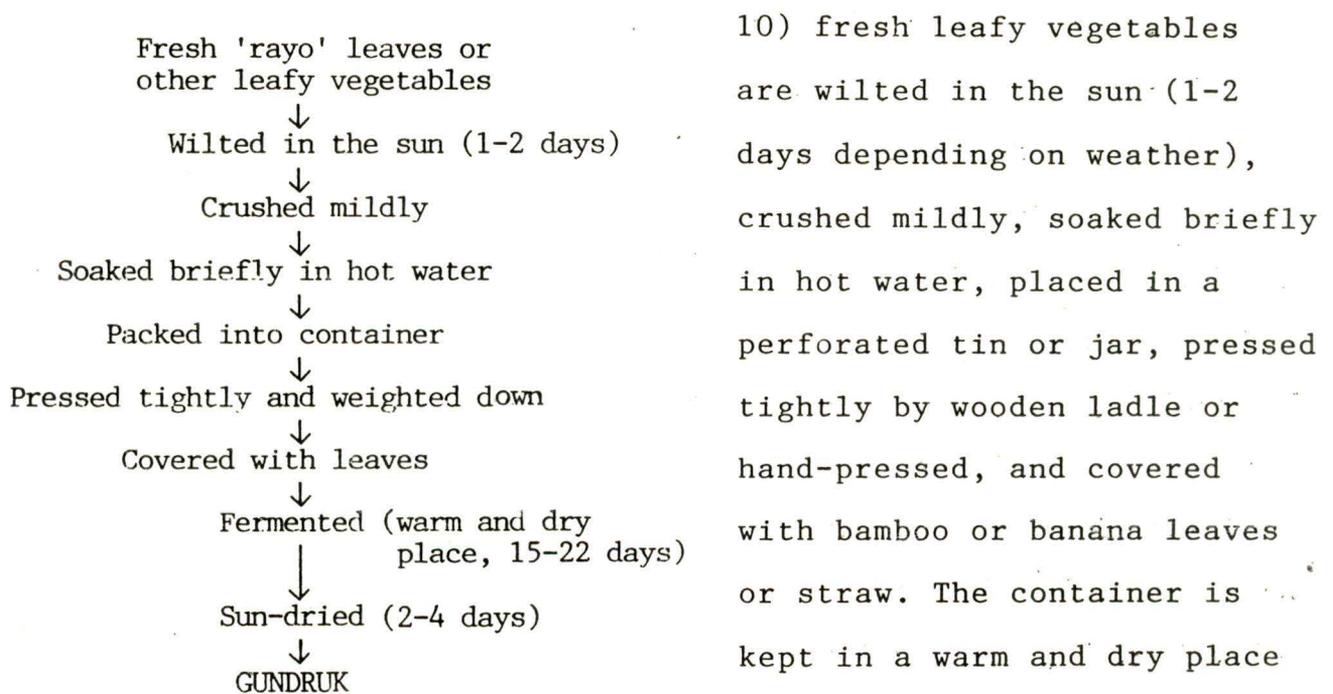


Fig. 9. Flow sheet of gundruk preparation. fermentation, determined by a typical flavour and sour taste, the fermented product is taken out

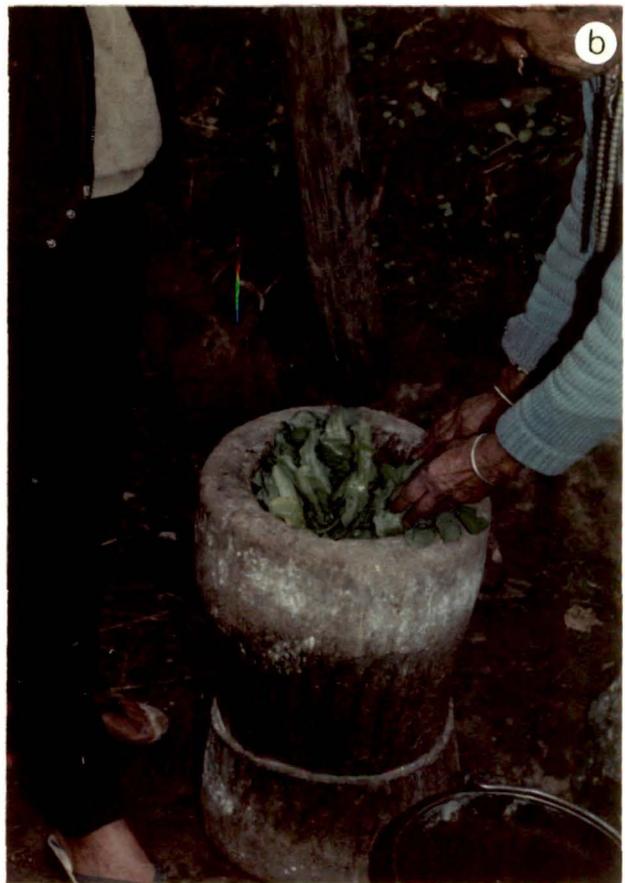


Fig. 10. A Nepali family preparing gundruk in a village of Kalimpong. (a) 'Rayo' leaves being wilted; (b) crushing leaves; (c) transferring the crushed leaves into a tin; and (d) leaving for fermentation

and dried in the sun for 2-4 days. Gundruk has a shelf-life of about one year. In a few places, a slight modification to the above method is practised. All the steps are identical to those of the above method, excepting that warm water (30-35°C) is frequently added to the perforated fermenting jar. After 3-7 days, they are taken out of the jar, squeezed and sun-dried.

Gundruk (Fig. 6b and 11) is typically used as a base for soup and as a pickle. The soup is made by soaking gundruk in water for about 2 min, squeezing out the liquid and frying along with salt, tomato, onion and green chilli. The fried mixture is boiled in rice water. The soup is served hot as an appetizer. Pickle is prepared by soaking gundruk in water, squeezing and mixing with salt, oil, onion and green chilli.

4.1.2.2. Sinki

Like kinema and gundruk, sinki was formerly confined to the Nepalis, but is now consumed by all the ethnic groups of these regions.

In the traditional method of its preparation (Fig. 12), fresh tap roots of radish (*Raphanus sativus* L.) are cleaned by washing, wilted for 1-2 days till they become soft, shredded, washed and placed tightly into earthen jar with the help of a wooden pestle. The jar is made air-tight by covering its mouth and kept in warm and dry place for 15-30 days. In some places, a pit of diameter and depth of about 1 m is dug in the ground and dried by fire. The pit is mud-plastered while hot and covered on all sides with dried leaves of bamboo, banana or radish. The shredded tap roots are placed in it, pressed tightly and covered with dried leaves. Heavy stones are placed to compress the substrate. The top of the pit is



Fig. 11. Sun-dried gundruk ready for marketing



Fig. 13. Freshly prepared (a) and sun-dried (b) sinki

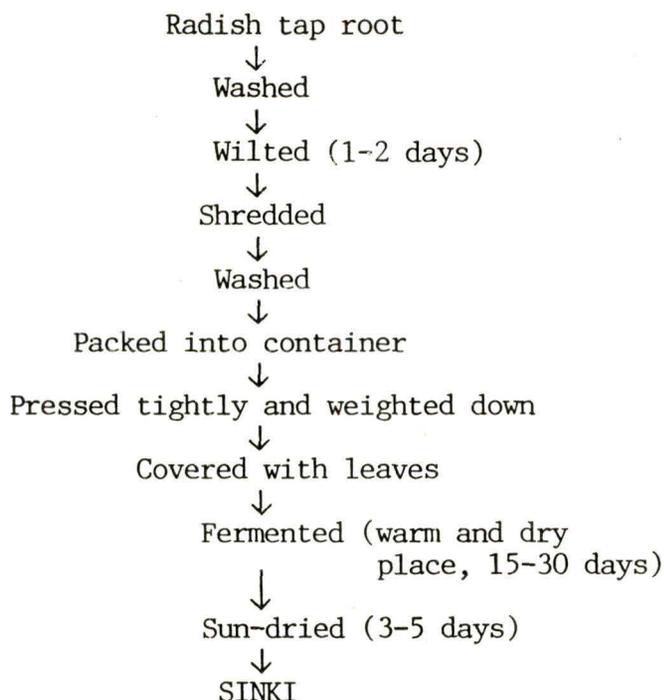


Fig. 12. Flow sheet of sinki preparation

4.1.2.3. Mesu

Mesu, a bamboo shoot pickle is common among the people inhabiting the bamboo-growing places of these regions. Mesu was originally

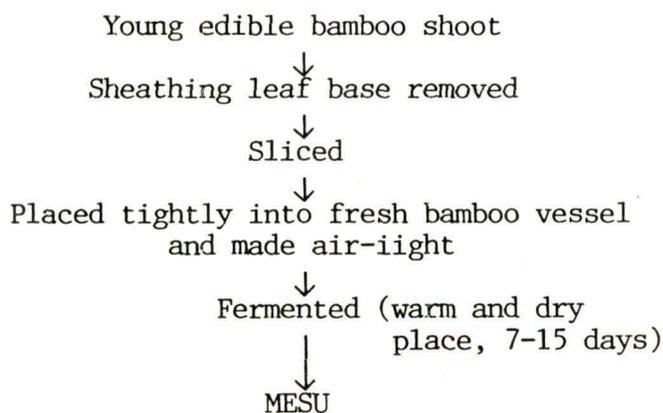


Fig. 14. Flow sheet of mesu preparation

in Fig. 14. Young edible bamboo ('choya bans' or 'tama',

again plastered with mud or cow dung and fermented for 30-40 days. The fermented mass is taken out and sun-dried (Fig. 6c and 13). The season of its preparation, its mode of consumption and its shelf-life are similar to those of gundruk.

confined to the Nepalis. The Lepchas and Bhutias call it 'sitit' and 'nyuk-ru', respectively. It is prepared in the months of June to September when young bamboo shoots are available.

The traditional method of mesu preparation is shown

Dendrocalamus hamiltonii Nees and Arnott; 'karati bans', *Bambusa tulda* Roxb.; 'bhalu bans', *Dendrocalamus sikkimensis* Gamble) shoots are chopped finely (1-1.5 cm x 0.3-0.7 cm x 0.3-0.7 cm) and pressed tightly into vessels, made from fresh hollow bamboo stems. The vessel is capped by leaves to provide an almost air-tight environment (Fig. 15). The material is allowed to ferment at ambient temperature (20-25°C) for 7-15 days. Completion of fermentation is indicated by the typical mesu flavour and taste. Fresh mesu (Fig. 16) has a shelf-life of about one week. However, mesu pickle can be stored for a year or more.

Mesu is commonly used as a pickle (Fig. 17) by mixing with salt, oil and green chillies. It is also used for preparing curry by frying and mixing with cooked meat.

4.1.2.4. Khalpi

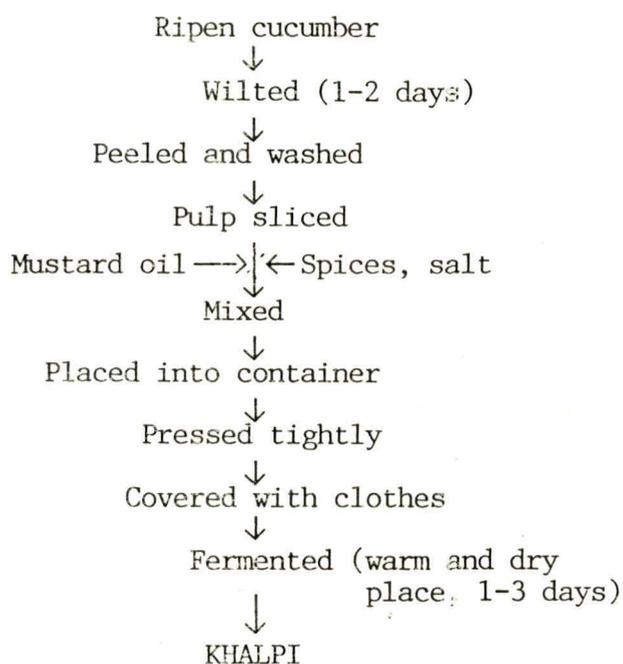


Fig. 18. Flow sheet of khalpi preparation

Khalpi is a salted cucumber pickle, prepared in a few cucumber-growing places of these regions. It is usually prepared during summer and early autumn.

In the traditional method of its preparation (Fig. 18), cucumber (*Cucumis sativus* L.), usually ripen, is wilted for 1-2 days, peeled, washed and sliced (5-7 cm x 2-3 cm x 2-3 cm). They are mixed with salt,

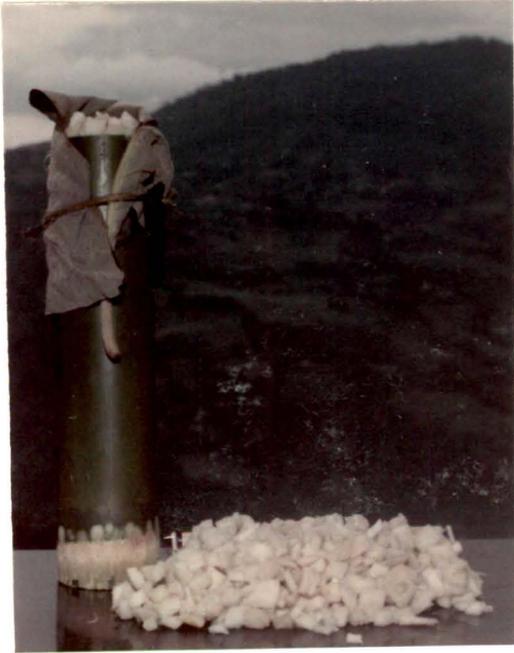


Fig. 15. Mesu along with a fermentation container

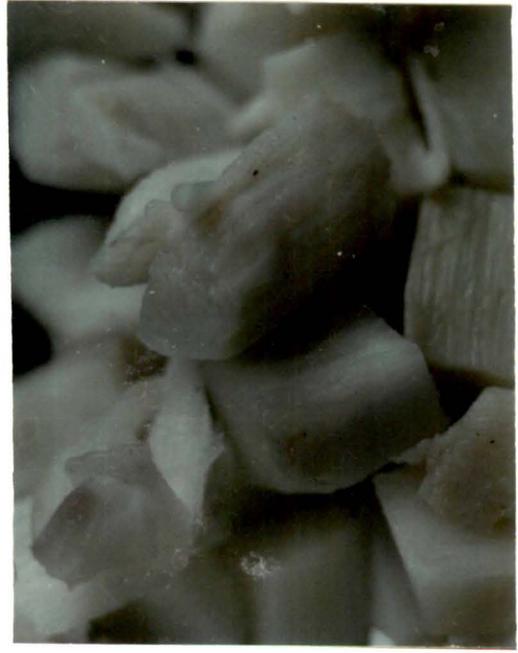


Fig. 16. Mesu (close view)



Fig. 17. Mesu pickle

spices and mustard oil, placed tightly into bamboo vessel or earthen jar, covered with clothes, kept at warm and dry place, and left to ferment for 1-3 days.

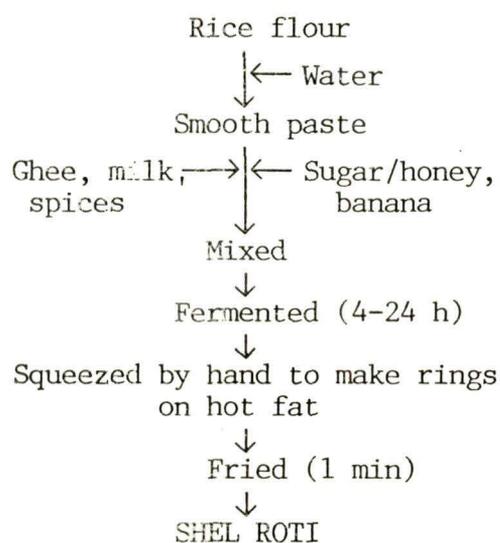
Khalpi used as a ready-made pickle, can be kept for 1-2 months.

4.1.3. Fermented cereal preparation

4.1.3.1. Shel roti

These are ring-shaped fried foods eaten traditionally by the Nepalis. Shel roti is prepared in almost every house during festivals.

Traditionally, they are prepared (Fig. 19) by mixing rice



flour paste (rice 1 kg and water 1 l) with banana (two small pieces), ghee (100 g), sugar (200 g) or honey (100 g), milk (500 ml) and some spices.

Sometimes, instead of banana, sodium bicarbonate (3 g) is added. The well-mixed batter is allowed to ferment for between 4 h

Fig. 19. Flow sheet of shel roti preparation

(during summer) and 24 h (during winter).

The leavened batter is squeezed by hand and deposited as continuous rings on hot fat (Fig. 20). These rings are fried and served hot. Shel roti is consumed as a confectionary bread (Fig. 21) with curry or pickle. It has a shelf-life of about one week.



Fig. 20. Fermented rice batter being squeezed by hand onto hot fat to prepare shell roti



Fig. 21. Shell roti as sold in market places

4.1.4. Fermented dairy products

4.1.4.1. Dahi

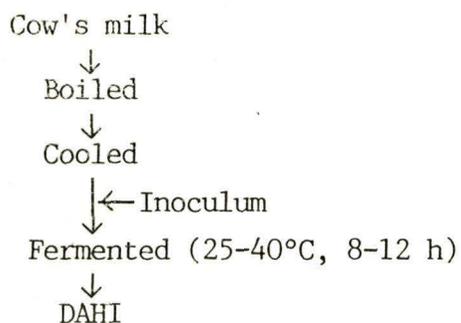


Fig. 22. Flow sheet of dahi preparation

Dahi ('sho', called by the Bhutias) is one of the major fermented dairy products commonly prepared in every house of these regions.

Traditionally, cow's milk is boiled, cooled, inoculated with previous batch of dahi

and allowed to ferment for 8-12 h at a warm place, by keeping near oven in kitchen (Fig. 22).

4.1.4.2. Kachcha churpi

Kachcha churpi ('chuiw', called by the Lepchas) is prepared (Fig. 23) from cow's milk. Dahi is churned in a bamboo vessel. Following

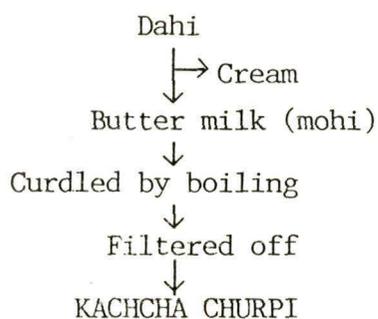


Fig. 23. Flow sheet of kachcha churpi preparation

separation of cream, the butter milk ('mohi') is curdled by boiling and filtered through a piece of muslin cloth. The coagulum is kept under pressure for 3-5 h to prepare kachcha churpi (Fig. 24). It can be kept for 2-3 days. Kachcha churpi is consumed as a condiment by mixing with sliced radish or cucumber, salt

and green chillies. It is also mixed with meat, vegetables and spices to prepare curry.



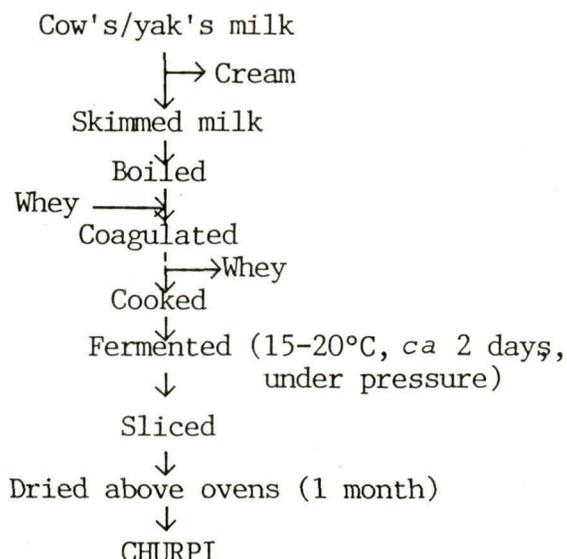
Fig. 24. Kachcha churpi being marketed in Kalimpong



Fig. 26. Dudh churpi (a) and churpi (b)

4.1.4.3. Churpi

Churpi ('chura', called by the Bhutias and 'khamum', by the Lepchas) is commonly prepared in high altitude areas (1300-1400 m) of Darjeeling hills and North and East Sikkim. Churpi is



prepared (Fig. 25) from cow's or yak's milk.

The cream is separated from milk by centrifugation and the skimmed milk is boiled and curdled by adding whey. After straining, the coagulum is cooked until the free water dries up. The highly stringy mass is wrapped with a cloth and cured at room temperature

Fig. 25. Flow sheet of churpi preparation

(15-20°C) for about two days under pressure of about 0.8-1.0 kg/cm², made with the aid of heavy stones. After pressing, the mass as such or after slicing is allowed to dry by keeping it above ovens in kitchen for about one month. The product becomes very hard and can be stored for a number of years. Churpi (Fig. 26b) is flat in taste and is used in much the same as chewing gum.

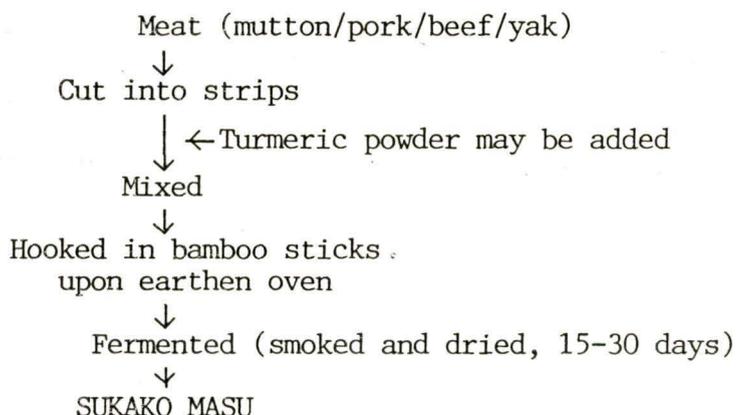
4.1.4.4. Dudh churpi

During the time of churpi making, the cooked, pressed and one week-dried coagulum is boiled in milk till the free milk dries up. It is again dried for about three weeks keeping in the sun or above ovens. Dudh churpi (Fig. 26a) is sweet and is used like churpi.

4.1.5. Fermented meat

4.1.5.1. Sukako masu

This smoked meat product ('sha-kampo', called by the Bhutias) is popular among the non-vegetarians of these regions. It is usually prepared during autumn to winter.



In the traditional method of its preparation (Fig. 27), mutton, pork, beef or yak's meat is cut into strips, may be mixed with turmeric, hooked in bamboo sticks, hanged over earthen oven in kitchen and allowed ferment by smoking and

Fig. 27. Flow sheet of sukako masu preparation

drying for 15-30 days. The shelf-life of sukako masu is 1-2 months. However, frequent sun-drying is required for prolonged (beyond 2 months) storage.

Sukako masu is consumed as a curry with rice. Curry is prepared by soaking sukako masu in water for a few min, squeezing out excess water and frying with salt, spices, onion and chillies with little water. Sometimes, it is cooked with vegetables.

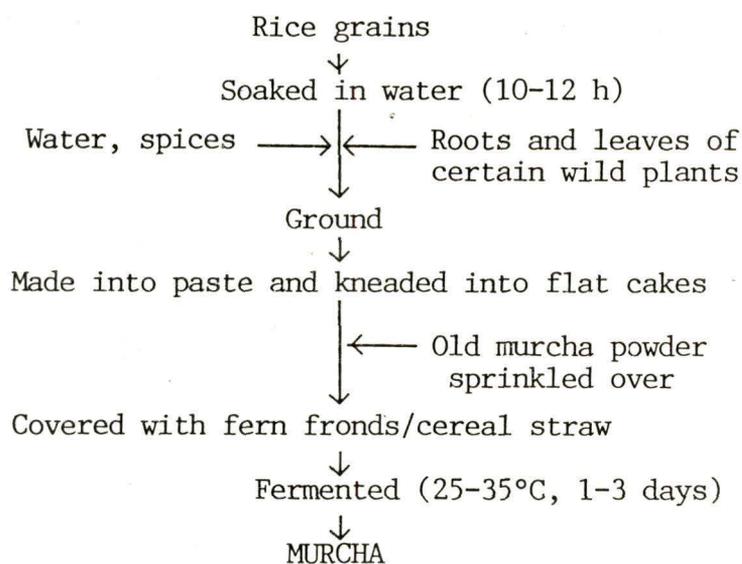
4.1.6. Starter culture

4.1.6.1. Murcha

Murcha is not a food but a starter culture used for several beverage fermentations, commonly consumed locally. 'Murcha' is a

Nepali word; the Lepchas use the word 'thamik', the Limbu (one of the ethnic groups of the Nepalis) call it 'khesung' and the Bhutias call it 'phab'. Trade in murcha is protected as a hereditary right of certain castes of the Nepalis (particularly Limbu and Rai) and the Lepchas.

Figure 28 illustrates the sequential addition of ingredients for murcha preparation. Rice (glutinous and non-scented), soaked



in water overnight (10-12 h), is placed in a large wooden mortar. A few pieces of the root of 'sweto-chitu' (*Plumbago zeylanica* L.), commonly used by the Lepchas of Bong Busty of Kalimpong), a few leaves of 'vimsen pathe' (*Buddleja asiatica* Lour.), and certain spices such as ginger

Fig. 28. Flow sheet of murcha preparation

and chilli are blended using a heavy-wooden, foot driven pestle (Fig. 29). Water is added to make dough which is hand-moulded into flattened cakes of 2-5 cm in diameter. Old murcha powder is sprinkled over the new cakes which are then wrapped in fern fronds (*Athyrium* sp.) with the fertile side (bearing the sori) touching the cakes. The cakes are placed above kitchen and covered successively with fresh fronds, dried fronds, straw and finally sackcloth. They take 1-3 days to dry at 25-35°C. This method is



Fig. 29. Ingredients (rice grains, rice flour, old murcha cakes, ginger, red chilli, leaves of 'vimsen pathe' and roots of 'sweto-chitu') used for murcha preparation



Fig. 30. Murcha cakes as marketed at Kalimpong

protected by the Limbu and the Rai communities. The Lepchas cover the cakes with rice straw instead of fern fronds. Cakes are dried in the sun and dry adhering fronds are removed. Murcha (Fig. 30) remains active for several months at room temperature and dry place.

4.1.7. Fermented beverages

4.1.7.1. Jnard

Jnard is a sweet, slightly acidic and alcoholic beverage prepared in almost every house of these regions. The word 'jnard', derived from the Mangaranti or Magar language (the Mangarantis or Magar being one of the castes of the Nepalis), is known by many synonyms ('chiang' by the Bhutias, 'chii' by the Lepchas, 'toongba' by the Nepalis; the word 'toongba' actually means the bamboo vessel in which jnard is consumed. Jnard is the fermented product of finger millet (*Eleusine coracana* Gaertn.), locally known as 'kodo' or 'marua', and is commonly cultivated in the Kalimpong area and at lower altitudes (up to 1300 m) in Sikkim.

In the traditional method of jnard preparation (Fig. 31), finger millet seeds are boiled in an open cooker for about 30 min, excess water is drained off and the boiled seeds are spread on leaves, preferably banana leaves. Murcha 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. They are then usually placed in an earthen jar and covered with leaves and cloth, and fermented in warm and dry place for 3-5 days after which a typical

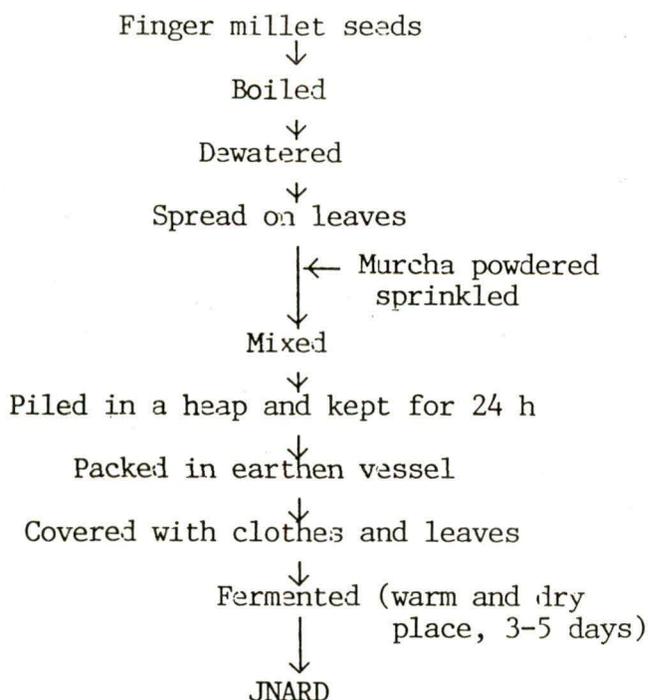


Fig. 31. Flow sheet of jnard preparation

flavour of jnard develops. In urban areas, the seeds are allowed to ferment in a polythene bag. If air is allowed access to the fermentation and sometimes even when the pots are kept air-tight, the product becomes sour.

Jnard is drunk by placing fermented seeds in a bamboo vassel (toongba) and warm water is added. After about 10

min, the beverage is ready to sip through a narrow bamboo straw (Fig. 32). This liquor is believed to be a good tonic, especially for post-natal women.

Although the term 'jnard' is commonly used for the finger millet beer, other substrates such as rice, maize, wheat, bajra, sweet potato, ginger, *Rhododendron* flower petals etc. are also used for jnard preparation. The names of the beers deriving from the raw materials used for fermentation are 'makai jnard' (maize beer), 'vate jnard' (rice beer) etc. The period of fermentation differs from substrate to substrate.

4.1.7.2. Raksi

Raksi ('arak', called by the Bhutias) is the distillate of rice beer, commonly used as a drink in these regions.



Fig. 32. Sipping jnard from toongba through a bamboo straw

4.1.8. Distribution of traditional fermented foods

Figures 33 and 34 show the distribution of traditional fermented foods in Darjeeling hills and Sikkim, respectively. About 90% of the inhabitants of these regions consume kinema, gundruk, sinki, shel roti, dahi, kachcha churpi, churpi, dudh churpi, sukako masu, jnard and raksi. Although mesu is consumed by the people of entire Kalimpong subdivision, its use is restricted to Mirik and adjoining areas only of Kurseong subdivision, and Bijanbari and Rimbik areas only of Darjeeling subdivision. In Sikkim, 60-70% of the population consume mesu. Consumption of masayura and khalpi is confined to a few pockets.

4.2. KINEMA

4.2.1. Proximate composition

The proximate composition of raw soya beans and kinema from different sources is presented in Table 8. The mean moisture content of soya beans was 10.8%, whereas that of kinema was 61.8%. Kinema had a very high protein content. The fat content in fermented soya beans was less than that of its raw material. Ash content of the laboratory-made kinema was relatively same as that of the substrate. However, market samples showed higher content of ash. The mean pH value of kinema was 7.89, whereas that of soya beans was 6.75. Titratable acidity and free fatty acidity of soya beans increased markedly after fermentation. The energy value of substrate is marginally higher than that of kinema.

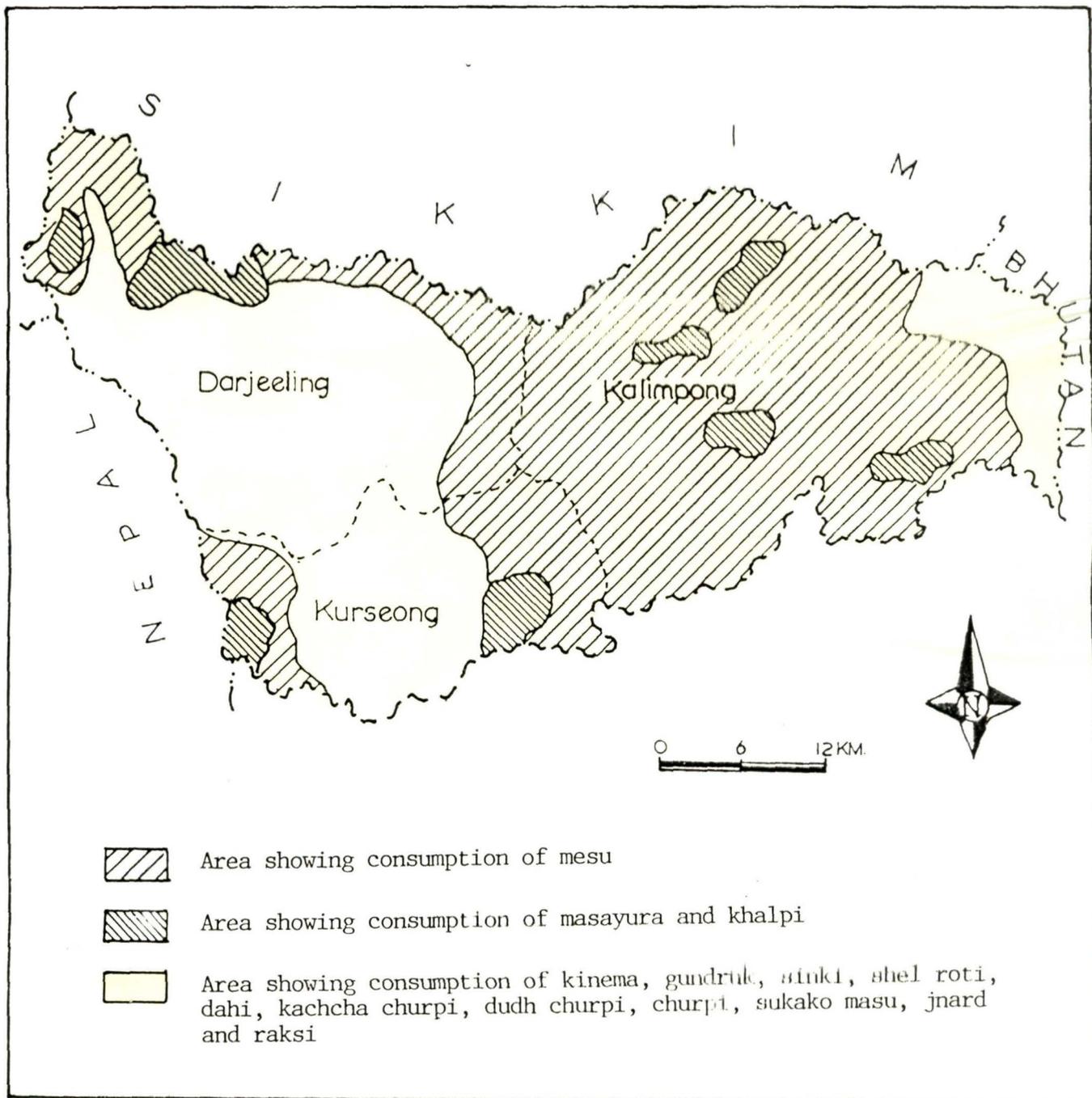


Fig. 33. Map showing distribution of consumption of traditional fermented foods in Darjeeling hills

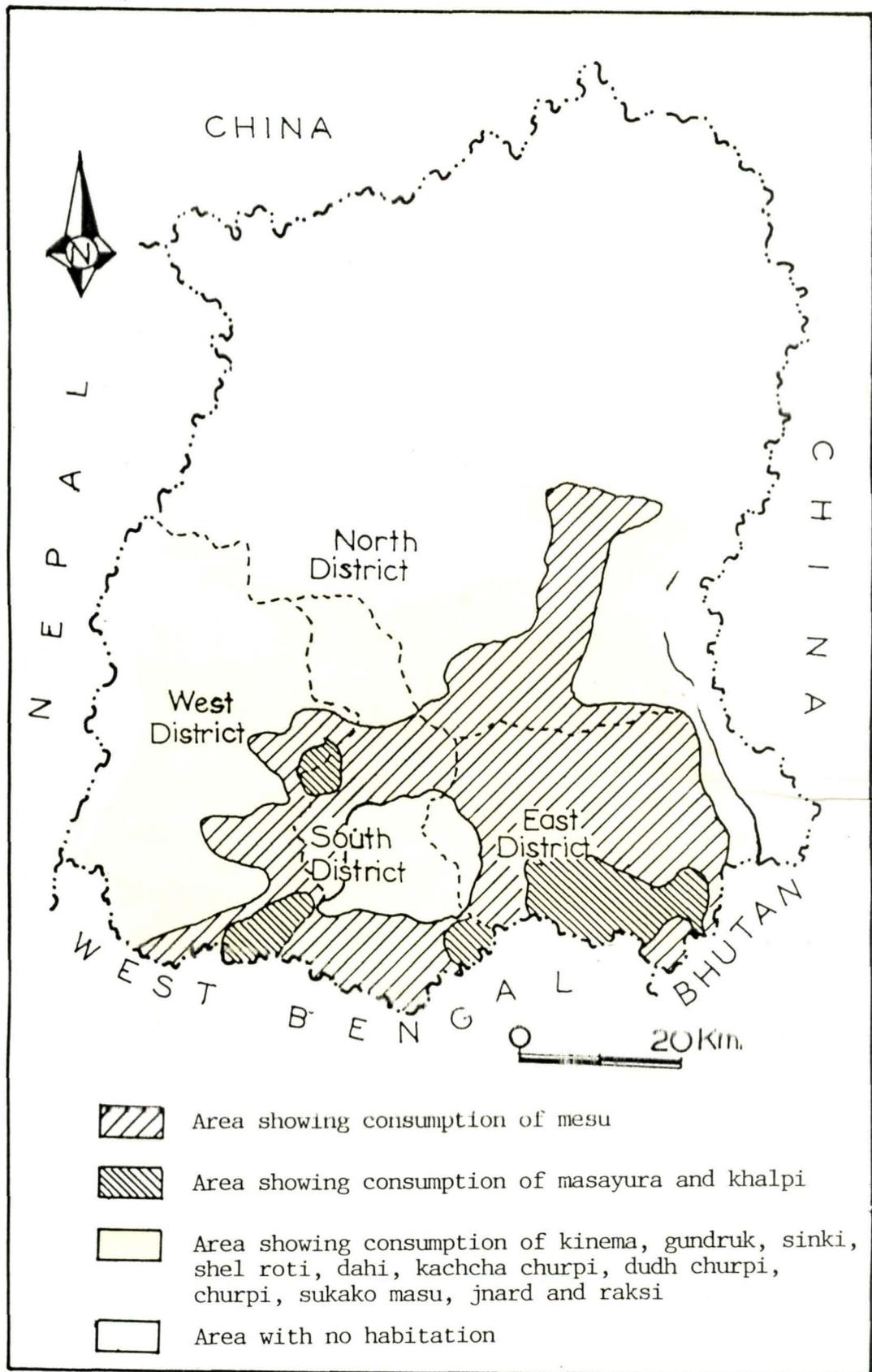


Fig. 34. Map showing distribution of consumption of traditional fermented foods in Sikkim

Table 8. Proximate composition of raw ingredient and kinema from different sources

Parameters	Soya beans	Kinema			
		Darjeeling market	Kalimpong market	Gangtok market	Laboratory-made
Moisture (%)	10.8 (9.5-12.0)	60.1 (57.3-61.4)	61.7 (58.5-64.4)	62.6 (59.5-64.1)	63.0 (61.7-64.8)
Protein (% DM) (Total N x 5.7)	47.1 (45.8-48.2)	47.3 (45.8-48.5)	47.8 (46.6-49.0)	48.0 (46.2-49.2)	48.7 (47.9-49.0)
Fat (% DM) (Ether extract)	22.1 (20.3-24.0)	16.3 (14.4-18.7)	16.8 (14.8-19.2)	17.7 (15.7-18.9)	16.1 (14.8-17.7)
Carbohydrate (% DM) (by difference)	25.8 (22.6-29.4)	29.0 (24.6-33.7)	28.0 (23.4-32.2)	27.4 (24.1-32.3)	29.6 (27.4-32.5)
Ash (% DM)	5.0 (4.5-5.2)	7.4 (6.1-8.2)	7.2 (6.4-8.4)	6.9 (5.8-7.8)	5.6 (4.8-5.9)
pH	6.75 (6.60-6.85)	7.94 (7.30-8.35)	7.82 (7.20-8.15)	7.71 (6.95-8.05)	8.10 (7.70-8.50)
Titratable acidity (as % lactic acid)	0.01 (0.01-0.01)	0.08 (0.06-0.09)	0.10 (0.06-0.10)	0.10 (0.07-0.12)	0.10 (0.09-0.10)
Free fatty acidity (as % linoleic acid)	0.08 (0.04-0.15)	3.0 (2.6-3.3)	2.7 (2.2-3.3)	2.6 (2.1-2.7)	2.4 (2.3-2.6)
Energy (MJ/100 g DM)	2.0 (1.9-2.2)	1.9 (1.7-2.1)	1.9 (1.7-2.1)	1.9 (1.8-2.1)	1.9 (1.8-2.0)

% DM, percentage on dry matter basis

Data represent the means of 10 samples. Ranges are given in parentheses.

4.2.2. Microbial analysis

4.2.2.1. Isolation of microorganisms from market and laboratory-made samples

Pure culture colonies developed on nutrient agar, MRS agar, GYP-CaCO₃ agar, APT agar, YM agar and PDA. Five hundred and two bacterial strains, isolated from 50 samples of kinema, were grouped on the basis of colony morphology, cell shape, spore formation, motility, catalase activity and oxygen requirement (Table 9). One hundred ninety eight yeast strains, isolated from the same 50 samples of kinema, were grouped on the basis of colony characters, cell morphology and mode of vegetative reproduction (Table 10). One representative strain from each group was selected randomly for identification.

4.2.2.2. Taxonomical studies

4.2.2.2.1. Bacteria

The colonies of the bacterial representative strains DK-W1, KK-W1, GK-W1, RK-W1 and LK-W1 were spreading white and fringed margin with dull surface. The cells of all these strains were motile, spore-forming, Gram positive rods (Fig. 35) showing catalase activity. The other group of the bacterial representative strains, DK-C1, KK-C1, GK-C1, RK-C1 and LK-C1 had white, small, isolated colonies, and were non-motile, nonspore-forming, Gram positive cocci in chains (Fig. 36). They were catalase negative and hydrolysed arginine.

Table 9. Selection of representative strains of bacteria isolated from kinema samples^a

Source	Number of strains ^b	Colony ^c	Cell shape	Spore formation	Motility	Catalase	O ₂ requirement	Grouped strains	Representative strains
Darjeeling market	98	Fds	Rod	+	+	+	Aerobic	60	DK-W1
		Ess	Coccus	-	-	-	Facultative	38	DK-C1
Kalimpong market	103	Fds	Rod	+	+	+	Aerobic	65	KK-W1
		Ess	Coccus	-	-	-	Facultative	38	KK-C1
Gangtok market	87	Fds	Rod	+	+	+	Aerobic	56	GK-W1
		Ess	Coccus	-	-	-	Facultative	31	GK-C1
Rongli market	109	Fds	Rod	+	+	+	Aerobic	62	RK-W1
		Ess	Coccus	-	-	-	Facultative	47	RK-C1
Laboratory-made	105	Fds	Rod	+	+	+	Aerobic	68	LK-W1
		Ess	Coccus	-	-	-	Facultative	37	LK-C1

^aNumber of samples was 10 from each source.

^bAll isolates were Gram positive.

^cFds, fringed with dull surface; Ess, entire with smooth surface

Table 10. Selection of representative strains of yeasts isolated from kinema samples^a

Source	Number of strains	Colony ^b	Cell shape	Mycelium	Vegetative reproduction ^c	Grouped strains	Representative strains
Darjeeling market	37	Cgs	Ellipsoidal	Pseudo	B(ml)	22	DK-Sml
		Wds	Cylindrical	True	S	15	DK-Chl
Kalimpong market	40	Cgs	Ellipsoidal	Pseudo	B(ml)	28	KK-Sml
		Wds	Cylindrical	True	S	12	KK-Chl
Gangtok market	43	Cgs	Ellipsoidal	Pseudo	B(ml)	30	GK-Sml
		Wds	Cylindrical	True	S	13	GK-Chl
Rongli market	45	Cgs	Ellipsoidal	Pseudo	B(ml)	27	RK-Sml
		Wds	Cylindrical	True	S	18	RK-Chl
Laboratory-made	33	Cgs	Ellipsoidal	Pseudo	B(ml)	33	LK-Sml

^aNumber of samples was 10 from each source.

^bCgs, cream coloured with glistening surface; Wds, white coloured with dull surface

^cB(ml), multilateral budding; S, splitting

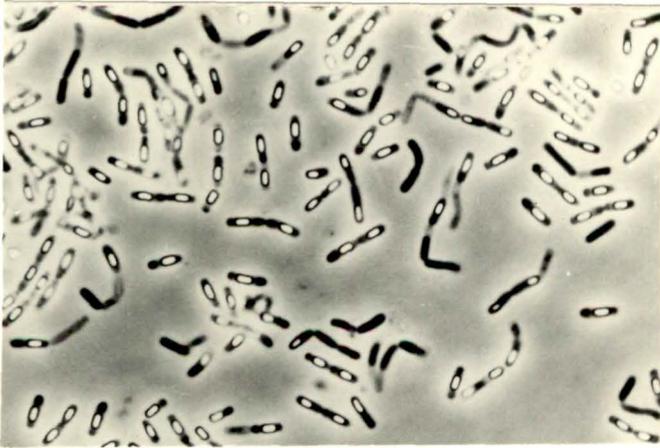


Fig. 35. *Bacillus subtilis* DK-W1 (NA, 18 h, 37°C) isolated from kinema, showing vegetative cells and endospores; phase contrast micrograph (x 1120)

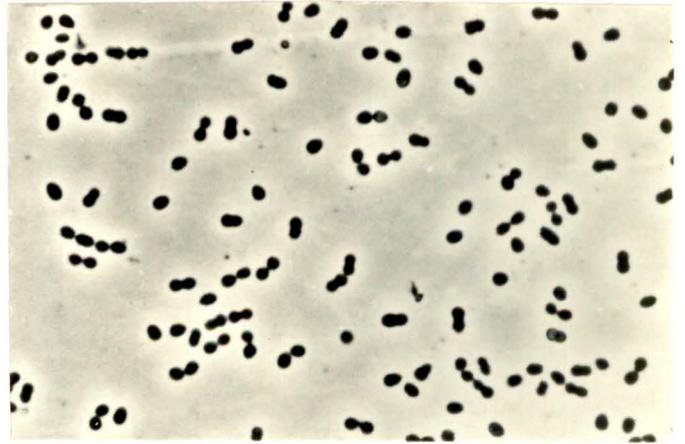


Fig. 36. *Enterococcus faecium* DK-C1 (MRS broth, 3 days, 37°C) isolated from kinema, showing coccal cells in chains; phase contrast micrograph (X 1120)

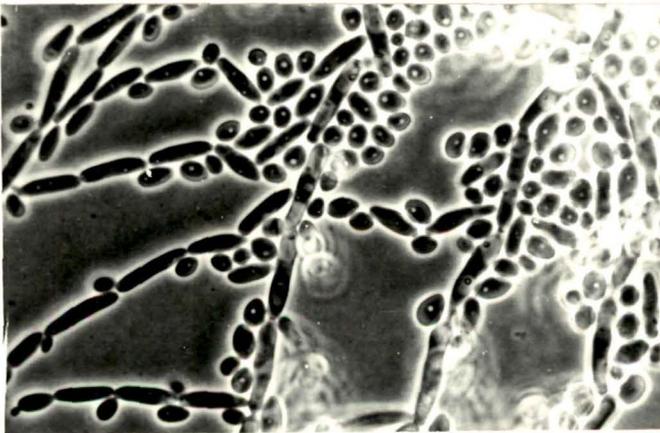


Fig. 37. *Candida parapsilosis* DK-Sml (Slide culture on PDA, 2 days, 28°C) isolated from kinema, showing ellipsoidal cells with pseudomycelia; phase contrast micrograph (x 800)

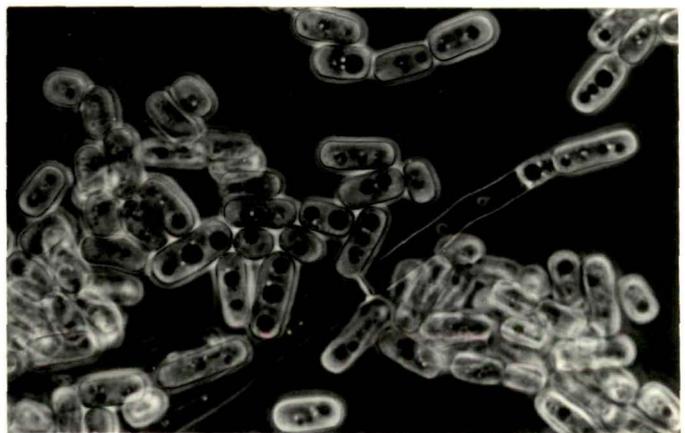


Fig. 38. *Geotrichum candidum* DK-Ch1 (Slide culture on PDA, 2 days, 28°C) isolated from kinema, showing cylindrical cells with true mycelia; phase contrast micrograph (x 800)

These characteristics showed that the strains DK-W1, KK-W1,, GK-W1, RK-W1 and LK-W1 belonged to the genus *Bacillus*, and the strains DK-C1, KK-C1, GK-C1, RK-C1 and LK-C1 belonged to the genus *Streptococcus*, as per the criteria laid down by Starr *et al.* (1981) and Sneath *et al.* (1986). The morphological and physiological characteristics of the representative strains of *Bacillus* and *Streptococcus* are shown in Tables 11 and 12, respectively.

Results for the API 50CH and API 20E tests were recorded following the manufacturer's interpretation table. On blood agar plates, a zone of greenish discoloration formed around the colonies whose margins were indistinct, indicating α -haemolytic activity of the streptococcal isolates.

Following the taxonomic keys of Norris *et al.* (1981) and Hardie (1986), the *Bacillus* strains were tentatively identified as *Bacillus subtilis* (Ehrenberg) Cohn and the *Streptococcus* strains were identified as *Streptococcus faecium* Orla-Jensen [*Enterococcus faecium* (Orla-Jensen) Schleifer and Klipper-Bälz].

4.2.2.2.2. Yeasts

The colonies of the yeast representative strains DK-Sm1, KK-Sm1, GK-Sm1, RK-Sm1 and LK-Sm1 were cream in colour with glistening surface and entire margin when grown on agar plates. However, they became mucoid without forming sediments in liquid medium. All these strains had ellipsoidal cells with multilateral budding and pseudomycelia (Fig. 37). Asci and ascospores were absent in these strains. The other group of the yeast representative strains DK-Ch1, KK-Ch1, GK-Ch1, RK-Ch1 and LK-Ch1 had white coloured

Table 11. Characteristics of representative strains of spore-forming bacteria isolated from kinema

Parameters	DK-W1	KK-W1	GK-W1	RK-W1	LK-W1
Morphological and supplementary tests					
Cell shape	Rod ^a	Rod	Rod	Rod	Rod
Cell width (μm)	$\emptyset.7-\emptyset.8$	$\emptyset.7-\emptyset.8$	$\emptyset.7-\emptyset.8$	$\emptyset.7-\emptyset.8$	$\emptyset.7-\emptyset.8$
Cell length (μm)	$2.\emptyset-4.1$	$2.\emptyset-4.2$	$2.\emptyset-4.\emptyset$	$2.\emptyset-4.1$	$2.\emptyset-4.\emptyset$
Motility	+	+	+	+	+
Spore shape	E-C ^b	E-C	E-C	E-C	E-C
Spore position	C/T ^c	C/T	C/T	C/T	C/T
Sporangium swollen	-	-	-	-	-
Gram reaction	+	+	+	+	+
Catalase	+	+	+	+	+
Anaerobic growth	-	-	-	-	-
Aerobic growth	+	+	+	+	+
Gas from glucose	-	-	-	-	-
Hydrolysis of					
Fat	+	+	+	+	+
Casein	+	+	+	+	+
Growth in NaCl					
5%	+	+	+	+	+
7%	+	+	+	+	+
Growth at					
10 and 15°C	-	-	-	-	-
20-55°C	+	+	+	+	+
60°C	-	-	-	-	-
Optimum temperature for growth (°C)	45	45	45	45	45

Parameters	DK-W1	KK-W1	GK-W1	RK-W1	LK-W1
Growth factor requirement	-	-	-	-	-
pH in VP broth <6.0	-	-	-	-	-
API 50CHB tests (Acid from)					
Glycerol	+	+	+	+	+
Erythritol	-	-	-	-	-
D-Arabinose	-	-	-	-	-
L-Arabinose	+	+	+	+	+
D-Ribose	+	+	+	+	+
D-Xylose	+	-	+	-	+
L-Xylose	-	-	-	-	-
Adonitol	-	-	-	-	-
β -Methyl-D-xyloside	-	-	-	-	-
D-Galactose	-	-	-	-	-
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	+
L-Sorbose	-	-	-	-	-
L-Rhamnose	-	-	-	-	-
Dulcitol	-	-	-	-	-
Inositol	+	+	+	+	+
Mannitol	+	+	+	+	+
Sorbitol	+	+	+	-	+
α -Methyl-D-mannoside	-	-	-	-	-
α -Methyl-D-glucoside	+	+	+	+	+
N-Acetylglucosamine	-	-	-	-	-
Amygdalin	-	-	-	-	-
Arbutin	-	-	-	-	-

Parameters	DK-W1	KK-W1	GK-W1	RK-W1	LK-W1
Esculin	+	+	+	+	+
Salicin	-	-	-	-	-
Cellobiose	-	-	-	-	-
Maltose	+	+	+	+	+
Lactose	-	-	-	-	-
Melibiose	+	+	+	+	+
Sucrose	+	+	+	+	+
Trehalose	+	+	+	+	+
Inulin	+	+	+	+	+
Melezitose	-	-	-	-	-
D-Raffinose	+	-	+	+	+
Starch	+	+	+	+	+
Glycogen	+	-	+	+	+
Xylitol	-	-	-	-	-
β -Gentibiose	-	-	-	-	-
D-Turanose	+	+	+	+	+
D-Lyxose	-	-	-	-	-
D-Tagatose	-	-	-	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
D-Arabitol	-	-	-	-	-
L-Arabitol	-	-	-	-	-
D-Gluconate	-	-	-	-	-
2-Ketogluconate	-	-	-	-	-
5-Ketogluconate	-	-	-	-	-

Parameters	DK-W1	KK-W1	GK-W1	RK-W1	LK-W1
API 20E tests					
ONPG (β -Galactosidase)	-	-	-	-	-
ADH (Arginine dihydrolase)	-	-	-	-	-
LDC (Lysine decarboxylase)	-	-	-	-	-
ODC (Ornithine decarboxylase)	-	-	-	-	-
CIT (Citrate utilization)	-	-	-	-	-
H ₂ S (H ₂ S production)	-	-	-	-	-
URE (Urease)	-	-	-	-	-
TDA (Tryptophan desaminase)	+	+	+	+	+
IND (Indole Production)	-	-	-	-	-
VP (Acetoin production)	+	+	+	+	+
GEL (Gelatin hydrolysis)	+	+	+	+	+
Oxidase	-	-	-	-	-
Reduction of nitrate to nitrite	-	-	-	-	-
Reduction of nitrite to N ₂	-	-	-	-	-

^aIn chains of 2-8 cells

^bEllipsoidal to cylindrical

^cCentral/Terminal

Table 12. Characteristics of representative strains of asporogenous bacteria isolated from kinema

Parameters	DK-C1	KK-C1	GK-C1	RK-C1	LK-C1
Morphological and supplementary tests					
Cell shape	Coccus ^a	Coccus	Coccus	Coccus	Coccus
Cell diameter (μm)	$\emptyset.3\emptyset-0.5\emptyset$	$\emptyset.35-0.52$	$\emptyset.3\emptyset-0.6\emptyset$	$\emptyset.4\emptyset-0.7\emptyset$	$\emptyset.3\emptyset-0.6\emptyset$
Motility	-	-	-	-	-
Gram reaction	+	+	+	+	+
Catalase	-	-	-	-	-
O ₂ requirement	F ^b	F	F	F	F
Gas from glucose	-	-	-	-	-
Hydrolysis of					
Fat	+	+	+	+	+
Casein	-	-	-	-	-
Gelatin	-	-	-	-	-
Arginine	+	+	+	+	+
Growth in 6.5% NaCl	+	+	+	+	+
Growth at pH 9.2 and 9.6	+	+	+	+	+
Growth in					
$\emptyset.1\%$ methylene blue	+	+	+	+	+
Growth at					
3 \emptyset -45°C	+	+	+	+	+
5 \emptyset °C	-	-	-	-	-
Optimum temperature					
for growth (°C)	37	37	37	37	37
Reduction of nitrate to nitrite	-	-	-	-	-

Parameters	DK-C1	KK-C1	GK-C1	RK-C1	LK-C1
Reduction of nitrite to N ₂	-	-	-	-	-
Indole production	-	-	-	-	-
VP (Acetoin production)	+	+	+	+	+
α-Haemolysis	+	+	+	+	+
β-Haemolysis	-	-	-	-	-
API 50CHS tests (Acid from)					
Glycerol	-	-	-	-	-
Erythritol	-	-	-	-	-
D-Arabinose	-	-	-	-	-
L-Arabinose	+	+	+	+	+
D-Ribose	+	+	+	+	+
D-Xylose	-	-	-	-	-
L-Xylose	-	-	-	-	-
Adonitol	-	-	-	-	-
β-Methyl-D-xyloside	-	-	-	-	-
D-Galactose	+	+	+	+	+
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	+
L-Sorbose	-	+	+	-	+
L-Rhamnose	-	-	-	-	-
Dulcitol	-	-	-	-	-
Inositol	-	-	-	-	-
Mannitol	+	+	+	+	+
Sorbitol	-	+	+	+	+
α-Methyl-D-mannoside	-	-	-	-	-
α-Methyl-D-glucoside	-	-	-	-	-

Parameters	DK-C1	KK-C1	GK-C1	RK-C1	LK-C1
N-Acetylglucosamine	+	+	+	+	+
Amygdalin	+	+	+	+	+
Arbutin	+	+	+	+	+
Esculin	+	+	+	+	+
Salicin	+	+	+	+	+
Cellobiose	+	+	+	+	+
Maltose	+	+	+	+	+
Lactose	+	+	+	+	+
Melibiose	+	+	+	+	+
Sucrose	+	+	+	+	+
Trehalose	+	+	+	+	+
Inulin	-	-	-	-	-
Melezitose	-	-	-	-	-
D-Raffinose	+	+	+	+	+
Starch	-	-	-	-	-
Glycogen	-	-	-	-	-
Xylitol	-	-	-	-	-
β -Gentibiose	+	+	+	+	+
D-Turanose	-	-	-	-	-
D-Lyxose	-	-	-	-	-
D-Tagatose	-	-	-	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
D-Arabitol	-	-	-	-	-
D-Gluconate	-	-	-	-	-

Parameters	DK-C1	KK-C1	GK-C1	RK-C1	LK-C1
2-Ketogluconate	-	-	-	-	-
5-Ketogluconate	-	-	-	-	-

^aIn chains of 2-10 cells

^bFacultative anaerobes

colonies with dull surface and fringed margin when grown on agar plates. These strains formed sediments when grown in liquid medium. The cells were cylindrical in shape (Fig. 38). Vegetative reproduction was by the formation of splitting cells. True mycelia were formed with arthroconidial development. None of these strains produced asci and ascospores. The detailed morphological and physiological characteristics of the representative strains of those yeasts were shown in Tables 13 and 14.

According to the criteria laid down by Barnett *et al.* (1983) and Kreger-van Rij (1984), the representative strains DK-Sm1, KK-Sm1, GK-Sm1, RK-Sm1 and LK-Sm1 were identified as *Candida parapsilosis* (Ashford) Langeron and Talice, and the strains DK-Ch1, KK-Ch1, GK-Ch1, RK-Ch1 and LK-Ch1 were identified as *Geotrichum candidum* Link.

4.2.2.3. Microbial load in substrate and products

The average microbial loads studied in 10 samples of kinema collected from each of Darjeeling, Kalimpong, Gangtok and Rongli

Table 13. Characteristics of representative strains of ellipsoidal-celled yeasts isolated from kinema

Parameters	DK-Sml	KK-Sml	GK-Sml	RK-Sml	LK-Sml
Morphological and supplementary tests					
Cell shape	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal	Ellipsoidal
Cell size					
Width (μm)	3.1-4.0	3.1-3.8	3.1-3.5	3.1-4.0	3.1-4.0
Length (μm)	5.7-9.6	5.7-9.2	5.7-9.0	5.7-9.2	6.7-9.6
Mycelium	Pseudo	Pseudo	Pseudo	Pseudo	Pseudo
Vegetative reproduction by	B(ml) ^a	B(ml)	B(ml)	B(ml)	B(ml)
Sexual reproduction	-	-	-	-	-
Reduction of nitrate to nitrite	-	-	-	-	-
Reduction of nitrite to N ₂	-	-	-	-	-
Hydrolysis of					
Fat	+	+	+	+	+
Urea	-	-	-	-	-
Growth at 37°C	+	+	+	+	+
Fermentation of					
D-Glucose	+	+	+	+	+
D-Galactose	-	-	-	-	-
Sucrose	-	-	-	-	-
Maltose	-	-	-	-	-
Lactose	-	-	-	-	-
D-Raffinose	-	-	-	-	-
Trehalose	-	-	-	-	-

Parameters	DK-Sml	KK-Sml	GK-Sml	RK-Sml	LK-Sml
API 50Ch tests					
(Growth and oxidation)					
Glycerol	+	+	+	+	+
Erythritol	-	-	-	-	-
D-Arabinose	-	-	-	-	-
L-Arabinose	-	-	-	+	-
D-Ribose	-	-	-	-	-
D-Xylose	-	+	-	+	-
L-Xylose	-	-	-	-	-
Adonitol	-	-	-	-	-
β -Methyl-D-xyloside	-	-	-	-	-
D-Galactose	+	+	+	+	-
D-Glucose	+	+	+	+	+
D-Fructose	+	+	+	+	+
D-Mannose	+	+	+	+	+
L-Sorbose	+	+	+	+	+
L-Rhamnose	-	-	-	-	-
Dulcitol	-	+	-	+	+
Inositol	-	-	-	-	-
Mannitol	-	-	-	+	-
Sorbitol	-	-	-	-	-
α -Methyl-D-mannoside	-	-	-	-	-
α -Methyl-D-glucoside	-	-	-	+	+
N-Acetylglucosamine	+	+	+	+	+
Amygdalin	-	-	+	-	+
Arbutin	-	+	-	-	+
Esculin	+	+	+	+	+
Salicin	-	-	-	-	-

Parameters	DK-Sml	KK-Sml	GK-Sml	RK-Sml	LK-Sml
Cellobiose	-	-	-	-	-
Maltose	-	+	-	+	-
Lactose	-	-	-	-	-
Melibiose	-	-	-	-	-
Sucrose	-	+	-	+	+
Trehalose	-	+	+	-	-
Inulin	-	-	-	-	-
Melezitose	-	-	-	-	-
D-Raffinose	-	-	-	-	-
Starch	-	-	-	-	-
Glycogen	-	-	-	-	-
Xylitol	-	+	-	+	-
Gentibiose	-	-	-	-	-
D-Turanose	-	-	-	-	-
D-Lyxose	-	-	-	-	-
D-Tagatose	-	-	-	-	-
D-Fucose	-	-	-	-	-
L-Fucose	-	-	-	-	-
D-Arabitol	-	-	-	-	-
L-Arabitol	-	-	-	-	-
D-Gluconate	-	-	-	-	-
2-Ketogluconate	-	+	-	-	+
5-Ketogluconate	-	-	-	-	-

^aMultilateral budding

Table 14. Characteristics of representative strains of cylindrical-celled yeasts isolated from kinema

Parameters	DK-Ch1	KK-Ch1	GK-Ch1	RK-Ch1
Morphological and supplementary tests				
Cell shape	Cylindrical	Cylindrical	Cylindrical	Cylindrical
Cell size				
Width (μm)	3.8- 4.6	3.8- 4.1	3.8- 4.0	3.8- 4.1
Length (μm)	7.8-15.2	7.6-15.0	7.6-14.9	7.6-15.2
Mycelium	True	True	True	True
Arthroconidia	+	+	+	+
Vegetative reproduction by	Splitting	Splitting	Splitting	Splitting
Sexual reproduction	-	-	-	-
Reduction of nitrate to nitrite	-	-	-	-
Reduction of nitrite to N_2	-	-	-	-
Hydrolysis of				
Fat	+	+	+	+
Urea	-	-	-	-
Growth at 37°C	+	+	+	+
Fermentation of				
D-Glucose	-	-	-	-
D-Galactose	-	-	-	-
Sucrose	-	-	-	-
Maltose	-	-	-	-
Lactose	-	-	-	-

Parameters	DK-Ch1	KK-Ch1	GK-Ch1	RK-Ch1
D-Raffinose	-	-	-	-
Trehalose	-	-	-	-
API 50CH tests				
(Growth and oxidation)				
Glycerol	+	+	+	+
Erythritol	-	-	-	-
D-Arabinose	-	-	-	-
L-Arabinose	-	-	-	-
D-Ribose	-	-	-	-
D-Xylose	+	+	+	+
L-Xylose	-	-	-	-
Adonitol	-	-	-	-
β -Methyl-D-xyloside	-	-	-	-
D-Galactose	+	+	-	+
D-Glucose	+	+	+	+
D-Fructose	-	-	-	-
D-Mannose	+	+	+	+
L-Sorbose	+	+	+	+
L-Rhamnose	-	-	-	-
Dulcitol	-	-	-	-
Inositol	-	-	-	-
Mannitol	+	+	+	+
Sorbitol	+	+	+	+
α -Methyl-D-mannoside	-	-	-	-
α -Methyl-D-glucoside	-	-	-	-
N-Acetylglucosamine	-	-	-	-
Amygdalin	-	-	-	-
Arbutin	-	-	-	-

Parameters	DK-Ch1	KK-Ch1	GK-Ch1	RK-Ch1
Esculin	+	+	+	+
Salicin	-	-	-	-
Cellobiose	-	-	-	-
Maltose	-	-	-	-
Lactose	-	-	-	-
Melibiose	-	-	-	-
Sucrose	-	-	-	-
Trehalose	-	-	-	-
Inulin	-	-	-	-
Melezitose	-	-	-	-
D-Raffinose	-	+	-	-
Starch	-	-	-	-
Glycogen	-	-	-	-
Xylitol	-	-	-	-
Gentibiose	-	-	-	-
D-Turanose	-	-	-	-
D-Lyxose	-	-	-	-
D-Tagatose	-	-	-	-
D-Fucose	-	-	-	-
L-Fucose	-	-	-	-
D-Arabitol	-	-	-	-
D-Gluconate	-	-	-	-
2-Ketogluconate	-	-	-	-
5-Ketogluconate	-	-	-	-

markets and of laboratory-made as well as soya bean [*Glycine max* (L.) Merrill cultivar 'local yellow'] seeds are shown in Table 15 and Fig. 39. In raw soya beans, while *Bacillus subtilis* was present at an average of 8×10^5 cfu/g, the other three types of microorganisms were absent. In kinema, the mean *Bacillus subtilis* count was 4.1×10^8 cfu/g, the mean *Enterococcus faecium* count was 7.6×10^7 cfu/g and the mean *Candida parapsilosis* count was 4.6×10^4 cfu/g. In the market samples, the mean *Geotrichum candidum* count was 1.9×10^4 cfu/g; however, it was absent in laboratory-made samples.

Table 16 shows the prevalence of various microorganisms in substrate and products, *Bacillus subtilis* was most prevalent, occurring in 100% samples of raw soya beans and of both market and laboratory-made kinema. Similarly, *Enterococcus faecium* was present in 100% samples of market and laboratory-made kinema. Yeast count in kinema was variable. *Candida parapsilosis* occurred in 60-80% of market samples and in 50% of laboratory-made samples. *Geotrichum candidum* was isolated from 40-50% of market samples.

4.2.3. Optimization of traditional process parameters

Table 17 shows the average sensory scores for optimizing aeration condition during fermentation of soya beans for kinema production. All the treatments differed significantly ($P < 0.05$) among themselves with respect to flavour, body and texture, colour and total score. The treatment B, perforated polythene bag for aeration condition, had the highest score compared to the treatment A, open polythene sheet, and the treatment C, tightly packed polythene bag. Hence, aeration condition during fermentation of soya beans for kinema

Table 15. Microbial load of raw ingredient and kinema from different sources

Microorganisms	Soya beans	cfu(x 10 ⁶)/g fresh weight				
		Kinema				
		Darjeeling market	Kalimpong market	Gangtok market	Rongli market	Laboratory-made
Bacteria						
<i>Bacillus subtilis</i>	0.8 (0.07-1.3)	322 (261-403)	405 (297-503)	438 (311-560)	430 (300-530)	460 (360-542)
<i>Enterococcus faecium</i>	0	54 (27-88)	68 (34-101)	92 (57-125)	80 (50-110)	85 (66-105)
Yeasts						
<i>Candida parapsilosis</i>	0	0.087 (0-0.175)	0.043 (0-0.090)	0.041 (0-0.085)	0.056 (0-0.100)	0.003 (0-0.007)
<i>Geotrichum candidum</i>	0	0.040 (0-0.081)	0.008 (0-0.025)	0.018 (0-0.055)	0.012 (0-0.035)	0

cfu, colony forming units

Data represent the means of 10 samples. Ranges are given in parentheses.

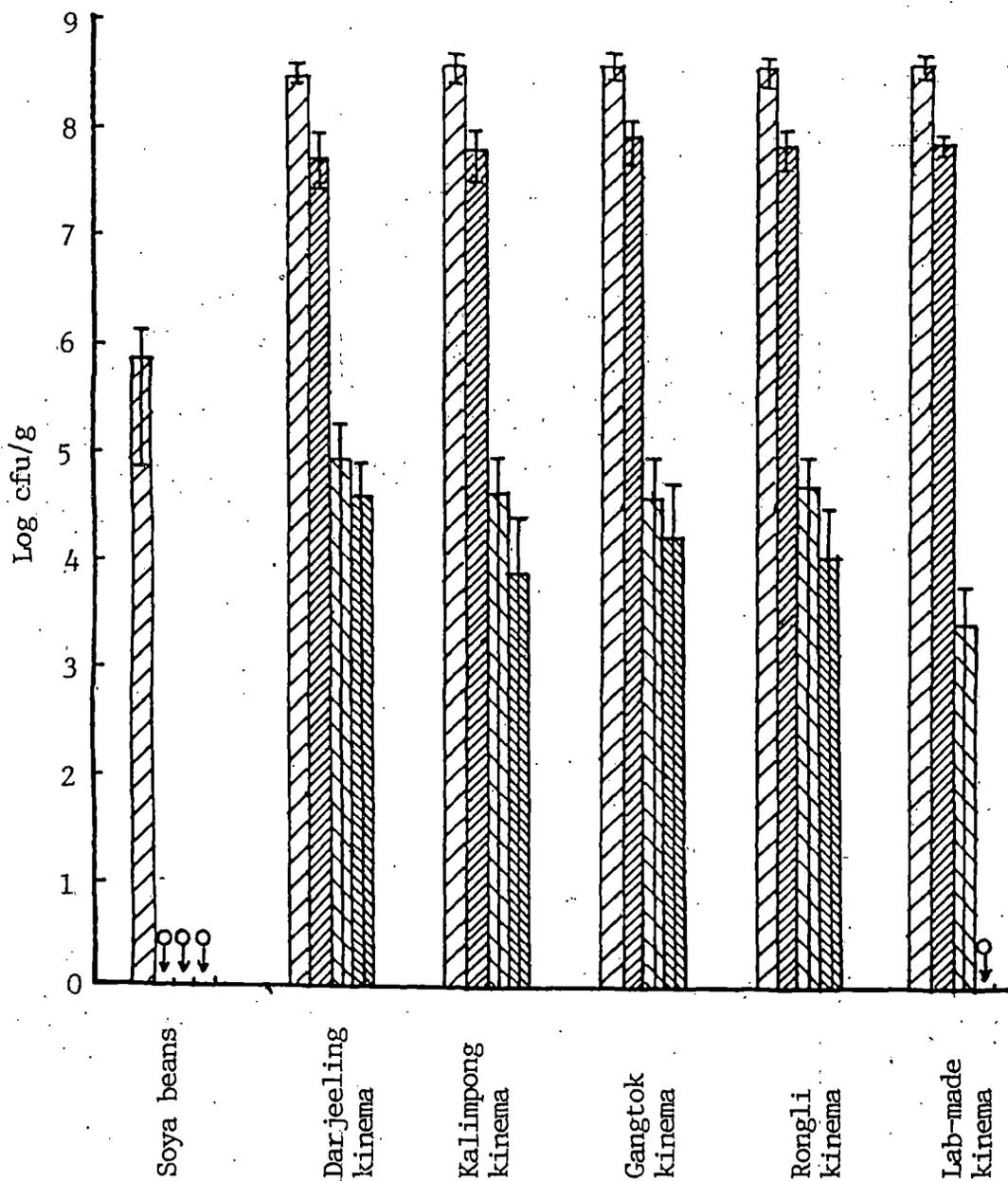


Fig. 39. Microbial load of raw ingredient and kinema from different sources. Values are the means with ranges of 10 samples.

Bacillus subtilis, ; *Enterococcus faecium*, ; *Candida parapsilosis*, ; *Geotrichum candidum*, 

Table 16. Prevalence of various microorganisms in raw ingredient and kinema from different sources

Microorganisms	Positive samples (%) ^a					
	Soya beans	Kinema				
		Darjeeling market	Kalimpong market	Gangtok market	Rongli market	Laboratory-made
Bacteria						
<i>Bacillus subtilis</i>	100	100	100	100	100	100
<i>Enterococcus faecium</i>	0	100	100	100	100	100
Yeasts						
<i>Candida parapsilosis</i>	0	80	60	70	60	50
<i>Geotrichum candidum</i>	0	50	40	40	40	0

^aExpressed on the basis of a total of 10 samples studied as in Table 15

Table 17. Average sensory scores for optimizing aeration condition during fermentation of soya beans for kinema preparation

Attributes	Aeration condition		
	A Open polythene sheet	B Perforated polythene bag	C Tightly packed polythene bag
Flavour	37.2 ^b (37.0-37.5)	43.1 ^a (42.9-43.3)	33.1 ^c (33.0-33.3)
Body and texture	35.3 ^b (35.0-35.5)	41.5 ^a (41.2-41.7)	32.1 ^c (32.0-32.2)
Colour	3.4 ^b (3.0-3.7)	4.0 ^a (4.0-4.0)	2.1 ^c (2.0-2.2)
Total score	75.9 ^b (75.0-76.7)	88.6 ^a (88.1-89.0)	67.3 ^c (67.0-67.7)

Cooking time in 0.7 kg/cm^2 steam pressure, 10 min; temperature of incubation, 37°C; fermentation time, 48 h

Data represent the means of three replications. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly ($P < 0.05$).

production was optimized at fermenting beans loosely packed with perforated polythene bag and then covered with clothes.

Table 18 reveals that all the treatments considered for optimizing wrapping material of soya beans differed significantly ($P < 0.05$) among themselves with respect to every sensory attribute. The treatment A, perforated polythene bag as a wrapping material, had the highest score compared to the other two treatments frequently used in the traditional process of kinema production. Hence, perforated polythene bag was the optimum wrapping material for kinema preparation.

All the treatments considered for optimizing cooking time of soya beans differed significantly ($P < 0.05$) among themselves with respect to every sensory attribute. (Table 19). The treatment B, cooking time of soya beans in 0.7 kg/cm^2 steam pressure for 10 min, had the highest score compared to the treatment C, 25 min cooking time and treatment A, 0 min. Hence, 10 min treatment in steam pressure was the optimum cooking time for kinema production.

All the treatments considered for optimizing temperature of incubation during soya bean fermentation differed significantly ($P < 0.05$) from each other in respect of flavour, body and texture, and total score, excepting treatments B and C which showed no significant difference ($P < 0.05$) in respect of colour (Table 20). The treatment B, temperature of incubation at 37°C during soya bean fermentation, had the highest score compared to the treatment C, 45°C and the treatment A, 28°C . Hence, 37°C was the optimum temperature for fermentation of soya beans during kinema production.

Table 21 shows that all the treatments considered for optimizing fermentation time during soya bean fermentation for

Table 18. Average sensory scores for optimizing wrapping materials of soya beans for kinema preparation

Attributes	Wrapping material		
	A Perforated polythene bag	B Leaves of <i>Ficus hookeriana</i>	C Leaves of ferns
Flavour	42.0 ^a (41.8-42.1)	35.8 ^c (35.7-36.0)	37.2 ^b (37.0-37.5)
Body and texture	40.8 ^a (40.7-41.0)	34.0 ^c (33.7-34.0)	38.0 ^b (37.9-38.1)
Colour	4.0 ^a (4.0-4.0)	2.7 ^c (2.5-3.0)	3.5 ^b (3.4-3.6)
Total score	86.8 ^a (86.5-87.1)	72.5 ^c (71.9-73.0)	78.7 ^b (78.3-79.2)

Aeration condition, loosely packed with packing materials and then covered with clothes; cooking time in 0.7 kg/cm² steam pressure, 10 min; temperature of incubation, 37°C; fermentation time, 48 h

Data represent the means of three replications. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly ($P < 0.05$).

Table 19. Average sensory scores for optimizing cooking time of soya beans for kinema preparation

Attributes	Cooking time in 0.7 kg/cm^2 steam pressure (min)		
	A 0	B 10	C 25
Flavour	34.1 ^c (33.8-34.5)	43.4 ^a (43.0-43.7)	35.2 ^b (35.0-35.5)
Body and texture	32.3 ^c (32.0-32.7)	39.6 ^a (39.5-39.7)	34.9 ^b (34.8-35.0)
Colour	2.0 ^c (2.0-2.0)	4.0 ^a (4.0-4.0)	3.3 ^b (3.0-3.5)
Total score	68.4 ^c (67.8-69.2)	87.0 ^a (86.5-87.4)	73.4 ^b (72.8-74.0)

Soya beans loosely packed with perforated polythene bag and then covered with clothes; temperature of incubation, 37°C; fermentation time, 48 h

Data represent the means of three replications. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly ($P < 0.05$).

Table 20. Average sensory scores for optimizing temperature of incubation during fermentation of soya beans for kinema preparation

Attributes	Temperature of incubation (°C)		
	A 28	B 37	C 45
Flavour	33.2 ^c (33.0-33.5)	42.2 ^a (42.0-42.5)	40.4 ^b (40.0-40.8)
Body and texture	31.0 ^c (31.0-31.1)	41.7 ^a (41.2-42.0)	38.2 ^b (38.0-38.5)
Colour	2.2 ^b (2.0-2.5)	4.0 ^a (4.0-4.0)	3.8 ^a (3.5-4.0)
Total score	66.4 ^c (66.0-67.1)	87.9 ^a (87.2-88.5)	82.4 ^b (81.5-83.3)

Soya beans loosely packed with perforated polythene bag and then covered with clothes; cooking time in 0.7 kg/cm^2 steam pressure, 10 min; fermentation time, 48 h

Data represent the means of three replications. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly ($P < 0.05$).

Table 21. Average sensory scores for optimizing fermentation time during fermentation of soya beans for kinema preparation

Attributes	Fermentation time (h)		
	A 24	B 48	C 72
Flavour	33.3 ^c (33.0-33.5)	44.1 ^a (44.0-44.2)	35.1 ^b (35.0-35.2)
Body and texture	32.0 ^c (31.5-33.0)	40.2 ^a (40.0-40.3)	34.9 ^b (34.8-35.1)
Colour	3.0 ^c (3.0-3.0)	4.0 ^a (4.0-4.0)	3.8 ^a (3.6-4.0)
Total score	68.3 ^c (67.5-69.5)	88.3 ^a (88.0-88.5)	73.8 ^b (73.4-74.3)

Soya beans loosely packed with perforated polythene bag and then covered with clothes; cooking time in 0.7 kg/cm^2 steam pressure, 10 min; temperature of incubation, 37°C

Data represent the means of three replications. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly ($P < 0.05$).

kinema production differed significantly ($P < 0.05$) with each other in respect of flavour, body and texture, and total score. However, in respect of colour, the treatments B and C showed no significant difference ($P < 0.05$). The treatment B, fermentation time of 48 h, had the highest total score compared to the treatment C, 72 h and the treatment A, 24 h. Hence, 48 h was the optimum time for fermentation of soya beans during kinema production.

4.2.4. Successional studies on soya beans during kinema production

Kinema was prepared in laboratory under the optimized conditions for studies on microbial and biochemical changes accompanying fermentation. Soya bean [*Glycine max* (L.) Merrill cultivar 'local yellow'] seeds were cleaned, washed, soaked in water for 8 h, dewatered and cooked with fresh water at 0.7 kg/cm² pressure for 10 min. Excess water was drained off and cooked beans were crushed lightly by a heavy wooden pestle to dehull and break the seeds. The whole soya bean grits were wrapped loosely in perforated polythene bags, covered with clothes and incubated at 37°C for 48 h. The successional studies were carried out at every 8 h intervals within a range of 0-48 h.

4.2.4.1. Microbial changes

Table 22 and Fig. 40 show the changes in microflora in soya beans during kinema production. Throughout fermentation, *Bacillus subtilis* was the most predominant microorganism. At the onset of fermentation, its level was about 10^6 /g fresh weight of the fermenting substrate. The number of this bacterium increased

Table 22. Changes in microbial load in soya beans during kinema preparation

Fermentation time (h)	cfu(x 10 ⁶)/g fresh weight		cfu(x 10 ²)/g fresh weight
	<i>Bacillus subtilis</i>	<i>Enterococcus faecium</i>	<i>Candida parapsilosis</i>
0	0.8 ^a (0.5-1.2)	0.02 ^a (0.007-0.04)	1.4 ^a (0.8-2.1)
8	5.2 ^b (3.7-7.8)	0.3 ^b (0.09-0.6)	2.5 ^b (1.5-3.9)
16	32.7 ^c (26.7-38.9)	2.0 ^c (1.3-3.3)	9.1 ^c (5.7-13.0)
24	95.8 ^d (67.3-120.1)	9.8 ^d (4.5-20.0)	13.2 ^d (9.2-18.1)
32	187.1 ^e (157.2-220.1)	22.2 ^e (14.2-35.1)	17.9 ^e (12.7-22.3)
40	330.4 ^f (277.4-375.4)	51.7 ^f (39.1-71.0)	20.7 ^e (16.4-29.0)
48	455.4 ^g (372.5-540.0)	82.3 ^f (65.7-98.8)	26.2 ^e (19.1-36.0)

cfu, colony forming units

Data represent the means of five batches of fermentation at 37°C. Ranges are given in parentheses.

Data were transformed into logarithmic values and then subjected to analysis of variance. Values bearing different superscripts in each column differ significantly (P<0.05).

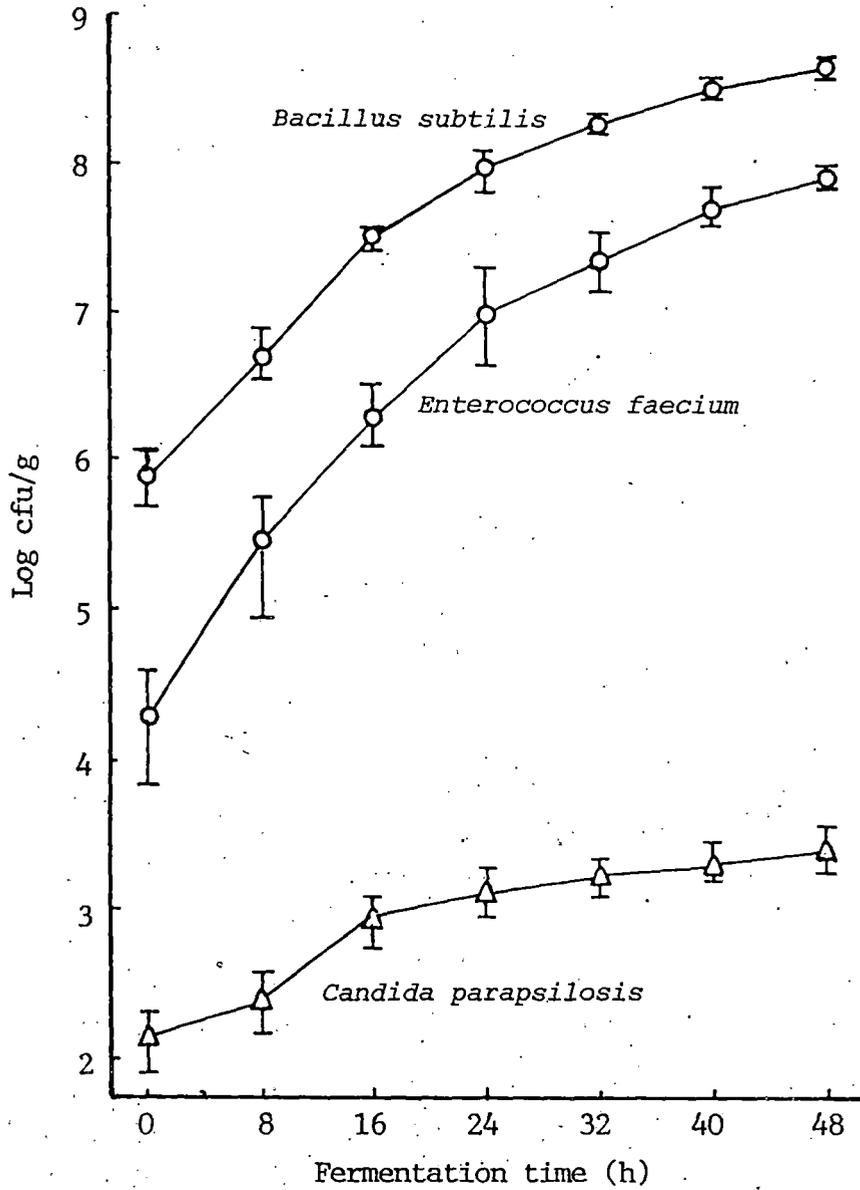


Fig. 40. Changes in microbial load in soya beans during kinema production. Values are the means of five batches of fermentation and bars represent ranges.

significantly ($P < 0.05$) at every 8 h intervals till the end of fermentation at 48 h. Although the initial load of *Enterococcus* was 40 times less than that of *Bacillus*, at the end of fermentation the number of the former was only 5 times less than the other. The increase in number of *Enterococcus* was significant ($P < 0.05$) at every 8 h intervals till the first 40 h of fermentation. The load of the only yeast, *Candida parapsilosis* recovered from the laboratory-made samples, was much less compared to the bacterial load. The number of the yeast cells increased throughout the fermentation period; till the first 32 h, the increase was significant ($P < 0.05$) at every 8 h intervals.

4.2.4.2. Biochemical changes

The biochemical changes in soya beans during kinema production are presented in Tables 23a and 23b. During the first 16 h of fermentation, the mean pH value declined significantly ($P < 0.05$) from 6.94 to 6.64. Then it increased sharply till the pH became 8.51 at 40 h (Table 23a and Fig. 41). However, after 40 h, the decline in pH was not significant ($P < 0.05$). The mean titratable acidity increased significantly ($P < 0.05$) at every 8 h intervals from 0.02 to 0.09% (Table 23a and Fig. 41). Since the 8th h of fermentation, the free fatty acid content increased significantly ($P < 0.05$) at every 8 h intervals (Table 23a and Fig. 41). The moisture content did not change appreciably (Table 23a). Interestingly, the increase in total nitrogen content of the fermentation product, over the raw beans, was significant ($P < 0.05$). Since the 8th h of fermentation, the protein nitrogen content of

Table 23a. Biochemical changes in soya beans during kinema production

Fermentation time (h)	pH	Titratable acidity (as % lactic acid)	Free fatty acidity (as % linoleic acid)	Moisture (%)
0	6.94 ^a (6.88-6.98)	0.020 ^a (0.017-0.023)	0.31 ^a (0.27-0.34)	61.91 ^{ab} (61.64-62.18)
8	6.80 ^b (6.69-6.90)	0.040 ^b (0.037-0.044)	0.34 ^a (0.31-0.38)	61.97 ^{be} (61.64-62.20)
16	6.64 ^c (6.57-6.69)	0.060 ^c (0.057-0.064)	0.61 ^b (0.54-0.70)	62.24 ^{ace} (61.71-62.64)
24	7.28 ^d (7.16-7.43)	0.065 ^d (0.060-0.069)	0.84 ^c (0.73-1.00)	62.51 ^{cd} (61.87-63.05)
32	7.91 ^e (7.70-8.12)	0.075 ^e (0.073-0.077)	1.30 ^d (1.23-1.38)	62.87 ^d (62.18-63.46)
40	8.51 ^f (8.45-8.55)	0.080 ^f (0.079-0.081)	1.77 ^e (1.69-1.84)	62.45 ^{cd} (62.10-62.68)
48	8.40 ^f (8.34-8.50)	0.090 ^g (0.088-0.092)	2.42 ^f (2.34-2.50)	62.26 ^{ace} (62.10-62.50)

Data represent the means of five batches of fermentation at 37°C. Ranges are given in parentheses.

Values bearing different superscripts in each column differ significantly (P<0.05).

Table 23b. Biochemical changes in soya beans during kinema production

Fermentation time (h)	Total nitrogen (% DM)	Protein nitrogen (% DM)	Non-protein nitrogen (% DM)	Soluble nitrogen (% DM)
0	8.50 ^a (8.48-8.52)	7.65 ^a (7.57-7.78)	0.85 ^a (0.70-0.95)	1.18 ^a (1.12-1.24)
8	8.50 ^a (8.49-8.52)	7.60 ^a (7.52-7.69)	0.90 ^a (0.80-1.00)	1.54 ^b (1.48-1.62)
16	8.51 ^{ab} (8.48-8.53)	7.38 ^b (7.35-7.40)	1.13 ^b (1.08-1.18)	1.99 ^c (1.90-2.10)
24	8.51 ^{ab} (8.49-8.54)	7.05 ^c (7.04-7.07)	1.46 ^c (1.42-1.50)	2.69 ^d (2.64-2.74)
32	8.53 ^{bc} (8.50-8.55)	6.73 ^d (6.70-6.75)	1.80 ^d (1.75-1.85)	3.43 ^e (3.28-3.55)
40	8.54 ^c (8.50-8.56)	6.19 ^e (6.12-6.24)	2.35 ^e (2.26-2.44)	4.52 ^f (4.46-4.59)
48	8.54 ^c (8.50-8.56)	5.99 ^f (5.90-6.05)	2.55 ^f (2.45-2.65)	5.04 ^g (4.98-5.13)

% DM, percentage on dry matter basis

Data represent the means of five batches of fermentation at 37°C.

Ranges are given in parentheses.

Values bearing different superscripts in each column differ significantly ($P < 0.05$).

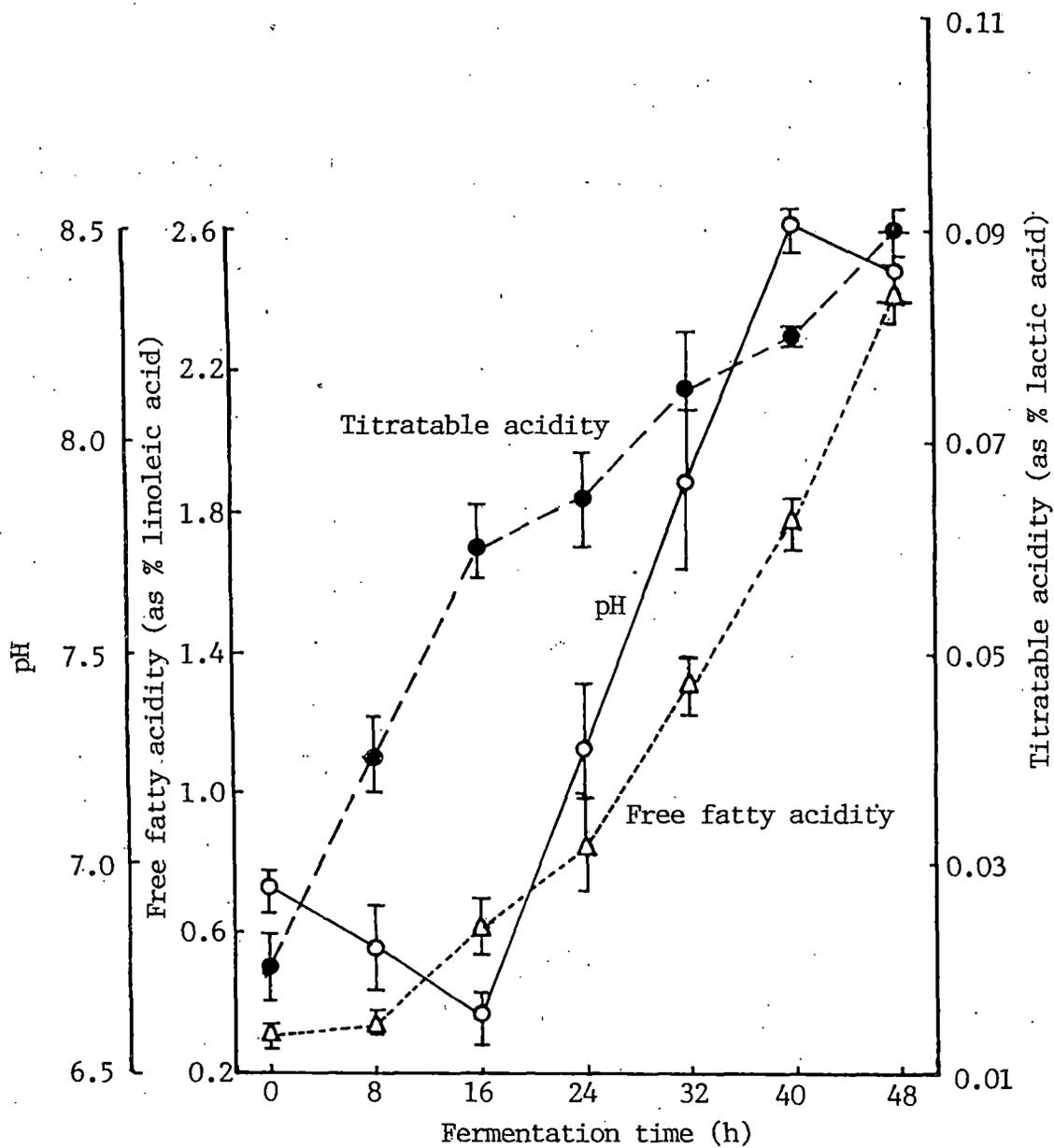


Fig. 41. Changes in pH and acidity in soya beans during kinema production at 37°C. Values are the means of five batches of fermentation and bars represent ranges. Where ranges overlap, bars for the lowest mean are shown to the left; and where three ranges overlap, bars for the highest mean are shown to the right.

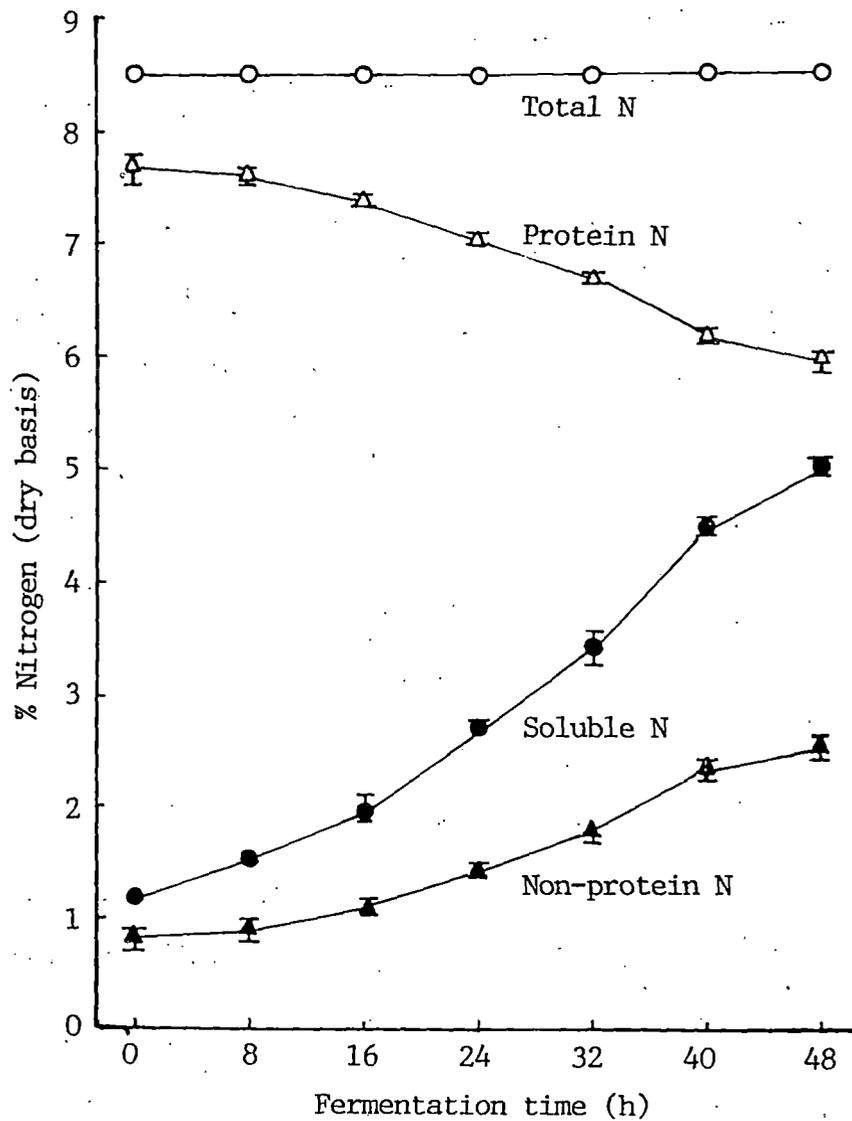


Fig. 42. Changes in nitrogen content in soya beans during kinema production at 37°C. Values are the means of five batches of fermentation and bars represent ranges.

of the beans decreased significantly ($P < 0.05$) at every 8 h intervals; on the other hand, there was significant ($P < 0.05$) increase in the non-protein nitrogen content. The increase in the soluble nitrogen content was significant ($P < 0.05$) at every 8 h intervals since the beginning of fermentation (Table 23b and Fig. 42).

4.2.5. Testing of the isolates for producing kinema

Sterilized soya beans were allowed to ferment with different combinations of *Bacillus subtilis*, *Enterococcus faecium*, *Candida parapsilosis* and *Geotrichum candidum*, isolated from traditionally prepared kinema (Table 24).

All the treatments differed significantly ($P < 0.05$) among themselves with respect to flavour, body and texture, and total score. However, in respect of colour, no significant difference ($P < 0.05$) was noticed among the treatments. The score in treatment A was the highest, suggesting *B. subtilis* as the best proven strain for production of kinema. Any microbial combination with this bacterium had an adverse effect on fermentation and consequent scoring (Table 24).

4.2.6. Growth of *Bacillus subtilis* DK-W1 at different oxygen levels

In an attempt to study the oxygen requirement during *Bacillus* fermentation of soya beans during kinema production, *B. subtilis* DK-W1 was grown under oxygen tensions. The organism did not grow at all under anaerobic condition. The counts obtained under aerobic as well as semi-anaerobic condition were the same 1.3×10^8

Table 24. Average sensory scores for testing the best proven strain(s) for kinema production

Attributes	Isolate(s)					
	A Bs	B Bs+Ef	C Bs+Ef+Cp	D Bs+Cp	E Bs+Ef+Cp+Gc	F Ef
Flavour	44.6 ^a (44.4-44.8)	38.9 ^b (38.8-39.0)	36.5 ^c (36.2-36.8)	33.8 ^d (33.7-33.9)	32.7 ^e (32.5-32.9)	30.0 ^f (30.0-30.1)
Body and texture	41.7 ^a (41.5-41.8)	38.1 ^b (38.0-38.2)	34.5 ^c (34.3-34.7)	31.6 ^d (31.5-31.6)	29.4 ^e (29.2-29.5)	27.1 ^f (27.0-27.1)
Colour	4.0 ^a (4.0-4.0)	4.0 ^a (4.0-4.0)	3.5 ^a (3.5-3.5)	3.0 ^a (3.0-3.0)	2.0 ^a (2.0-2.0)	2.0 ^a (2.0-2.0)
Total score	90.3 ^a (90.1-90.6)	81.0 ^b (80.0-81.2)	74.5 ^c (74.0-75.0)	68.4 ^d (68.2-68.5)	64.1 ^e (63.9-64.4)	59.1 ^f (59.0-59.2)

Bs, *Bacillus subtilis*; Ef, *Enterococcus faecium*; Cp, *Candida parapsilosis*; Gc, *Geotrichum candidum*

Data represent the means of three batches of fermentation at 37°C for 24 h. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly (P<0.05).

(range, 0.1-3.0) cfu/g fresh kinema, giving virtually the same growth. The organism, however, spread better under semi-anaerobic condition showing larger colony sizes.

4.2.7. Improvement of the production of kinema by pure culture fermentation

Attempts were made to improve the production of kinema by inoculating *Bacillus subtilis* DK-W1, the best proven kinema producing strain isolated from kinema sample of Darjeeling market. The period of fermentation of soya beans in monoculture inoculation was optimized (Table 25). It revealed that all the treatments differed significantly ($P < 0.05$) among themselves in respect of flavour, body and texture and total score. However, in respect of colour, there was no significant difference ($P < 0.05$) among the treatments A, B and C, treatments D, E and I, and treatments F, G and H. Treatment G, the fermentation time of 18 h, had the highest score in respect of flavour, body and texture and total score. In respect of colour, treatments F, G and H had the same score which was higher compared to the other treatments. Hence, the 18 h period at 45°C was found to be optimum for soya bean fermentation by *B. subtilis* only.

4.2.8. Successional studies on soya beans during monoculture fermentation

Kinema prepared by inoculating sterilized soya beans with *Bacillus subtilis* DK-W1 was studied at every 3 h intervals within a range of 6-18 h.

Table 25. Average sensory scores for optimizing the fermentation time of kinema production following monoculture (*Bacillus subtilis* DK-W1) inoculation of soya beans

Attributes	Fermentation time (h)								
	A 6	B 8	C 10	D 12	E 14	F 16	G 18	H 20	I 22
Flavour	34.9 ⁱ (34.8-35.0)	35.4 ^h (35.2-35.5)	36.7 ^g (36.5-36.8)	38.8 ^f (38.8-38.9)	42.6 ^{de} (42.5-42.7)	45.6 ^b (45.5-45.7)	46.7 ^a (46.5-46.8)	44.1 ^c (44.0-44.2)	42.2 ^e (42.1-42.4)
Body and texture	33.5 ^h (33.5-33.6)	34.4 ^g (34.2-34.5)	36.9 ^f (36.8-36.9)	38.0 ^e (38.0-38.1)	41.7 ^c (41.5-41.8)	42.5 ^b (42.4-42.5)	43.2 ^a (43.0-43.4)	42.4 ^b (42.3-42.5)	41.2 ^d (41.0-41.3)
Colour	2.5 ^c (2.4-2.5)	2.5 ^c (2.4-2.5)	2.5 ^c (2.5-2.5)	3.5 ^b (3.4-3.5)	3.5 ^b (3.5-3.5)	4.0 ^a (4.0-4.0)	4.0 ^a (4.0-4.0)	4.0 ^a (4.0-4.0)	3.6 ^b (3.5-3.8)
Total score	70.9 ⁱ (70.7-71.1)	72.3 ^h (71.8-72.5)	76.1 ^g (75.8-76.2)	80.3 ^f (80.2-80.5)	87.8 ^d (87.5-88.0)	92.1 ^b (91.9-92.2)	93.9 ^a (93.5-94.2)	90.5 ^c (90.3-90.7)	87.0 ^e (86.6-87.5)

Data represent the means of three batches of fermentation at 45°C. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly ($P < 0.05$).

4.2.8.1. Microbial changes

The load of *Bacillus subtilis* DK-W1 increased from 10^5 cfu/g fresh weight of soya beans at 0 h to 10^9 cfu/g fresh weight of kinema at 18h. During the first 15 h of fermentation, its increase was significant ($P < 0.05$) at every sampling time intervals (Table 26 and Fig. 43).

4.2.8.2. Biochemical changes

Tables 27a and 27b show the biochemical changes in soya beans during monoculture fermentation with *Bacillus subtilis* DK-W1 for kinema production. During fermentation, the pH value initially dropped down significantly ($P < 0.05$) from 6.9 at 0 h to 6.4 at 6 h, and then increased significantly ($P < 0.05$) at every 3 h intervals until the pH reached 7.5 at 18 h (Fig. 44). The titratable acidity and free fatty acidity (Fig. 44) continued to increase significantly ($P < 0.05$) at every 3 h intervals. The moisture content remained relatively constant throughout the fermentation. However, there was significant increase ($P < 0.05$) in the total nitrogen content of the product over the beans at 0 h of fermentation. While the protein nitrogen content in the fermenting mass had a significant ($P < 0.05$) decrease, the non-protein as well as soluble nitrogen contents increased significantly ($P < 0.05$) at every 3 h intervals till the end of fermentation at 18 h (Fig. 45).

4.3. SINKI

4.3.1. Proximate composition

The proximate composition of the substrate and the fermented products from different sources is presented in Table 28. The mean

Table 26. Changes in the microbial load during soya bean fermentation with *Bacillus subtilis* DK-W1 inoculation for kinema production

Fermentation time (h)	cfu($\times 10^8$)/g fresh weight
0	0.003 ^a (0.001-0.005)
6	0.20 ^b (0.15-0.28)
9	1.7 ^c (1.2-2.3)
12	8.1 ^d (7.0-10.2)
15	18.5 ^e (16.3-21.0)
18	29.2 ^e (26.7-33.1)

cfu, colony forming units

Data represent the means of three batches of fermentation at 45°C. Ranges are given in parentheses.

Data were transformed into logarithmic values and then subjected to analysis of variance. Values bearing different superscripts differ significantly ($P < 0.05$).

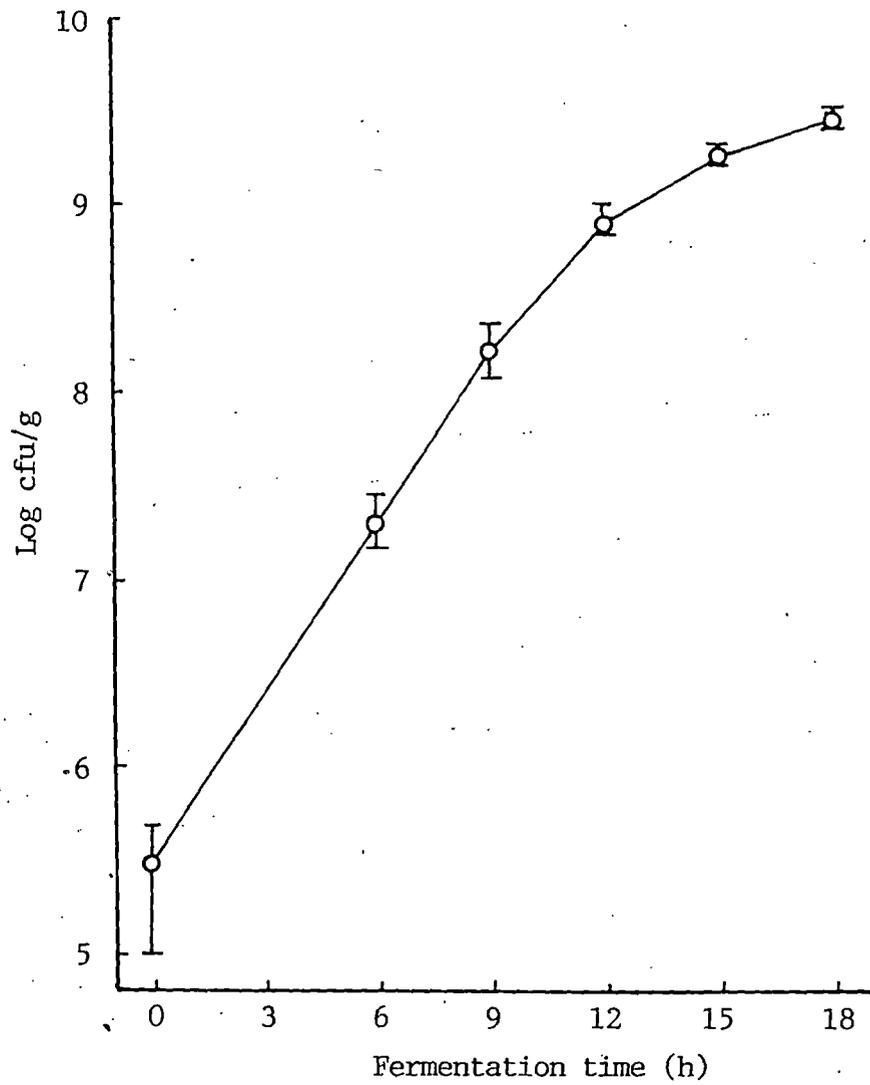


Fig. 43. Change in the load of *Bacillus subtilis* DK-W1 in soya beans during monoculture fermentation for kinema production at 45°C. Values are the means of three batches of fermentation and bars represent ranges.

Table 27a. Biochemical changes in soya beans during monoculture fermentation with *Bacillus subtilis* DK-W1 for kinema production

Fermentation time (h)	pH	Titrateable acidity (as % lactic acid)	Free fatty acidity (as % linoleic acid)	Moisture (%)
0	6.91 ^a (6.88-6.94)	0.020 ^a (0.017-0.024)	0.31 ^a (0.27-0.34)	62.00 ^a (61.85-62.10)
6	6.41 ^b (6.38-6.44)	0.040 ^b (0.038-0.042)	0.38 ^b (0.34-0.42)	62.03 ^{ab} (61.85-62.13)
9	6.52 ^c (6.46-6.58)	0.050 ^c (0.048-0.052)	0.61 ^c (0.58-0.65)	62.10 ^{bc} (61.90-62.25)
12	6.88 ^{ad} (6.84-6.91)	0.070 ^d (0.068-0.072)	0.73 ^d (0.69-0.77)	62.17 ^{cd} (62.00-62.30)
15	7.10 ^e (7.00-7.22)	0.080 ^e (0.077-0.083)	1.00 ^e (0.92-1.07)	62.24 ^d (62.02-62.40)
18	7.50 ^f (7.46-7.55)	0.095 ^f (0.093-0.097)	1.19 ^f (1.15-1.23)	62.24 ^d (62.04-62.36)

Data represent the means of three batches of fermentation at 45°C. Ranges are given in parentheses.

Values bearing different superscripts in each column differ significantly ($P < 0.05$).

Table 27b. Biochemical changes in soya beans during monoculture fermentation with *Bacillus subtilis* DK-W1 for kinema production

Fermentation time (h)	Total nitrogen (% DM)	Protein nitrogen (% DM)	Non-protein nitrogen (% DM)	Soluble nitrogen (% DM)
0	8.50 ^a (8.48-8.52)	7.74 ^a (7.69-7.78)	0.76 ^a (0.70-0.83)	1.20 ^a (1.12-1.27)
6	8.50 ^a (8.48-8.52)	7.49 ^b (7.48-7.51)	1.01 ^b (0.97-1.04)	1.54 ^b (1.46-1.61)
9	8.51 ^{ab} (8.49-8.52)	7.15 ^c (7.12-7.19)	1.36 ^c (1.30-1.40)	2.27 ^c (2.17-2.36)
12	8.52 ^{ab} (8.49-8.54)	6.80 ^d (6.71-6.89)	1.72 ^d (1.60-1.83)	2.80 ^d (2.75-2.89)
15	8.53 ^b (8.50-8.55)	6.51 ^e (6.41-6.60)	2.02 ^e (1.90-2.14)	3.55 ^e (3.40-3.66)
18	8.53 ^b (8.50-8.55)	6.30 ^f (6.24-6.32)	2.23 ^f (2.18-2.31)	4.17 ^f (4.11-4.25)

% DM, percentage on dry matter basis

Data represent the means of three batches of fermentation at 45°C.

Ranges are given in parentheses.

Values bearing different superscripts in each column differ significantly (P<0.05).

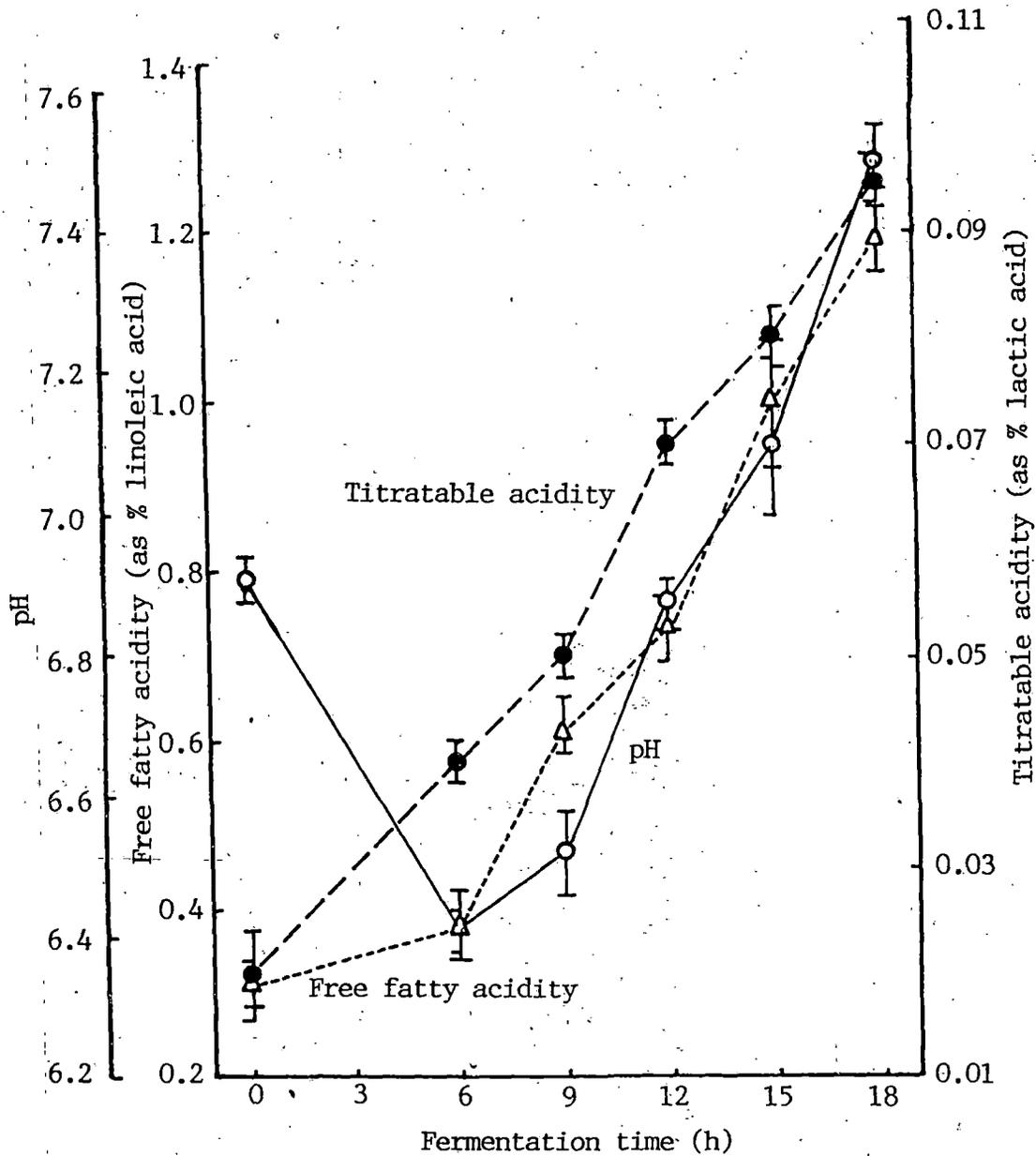


Fig. 44. Changes in pH and acidity in soya beans during monoculture fermentation with *Bacillus subtilis* DK-W1 for kinema production at 45°C. Values are the means of three batches of fermentation with ranges. Where ranges overlap, bars for the lowest mean are shown to the left; and where three ranges overlap, bars for the highest mean are shown to the right.

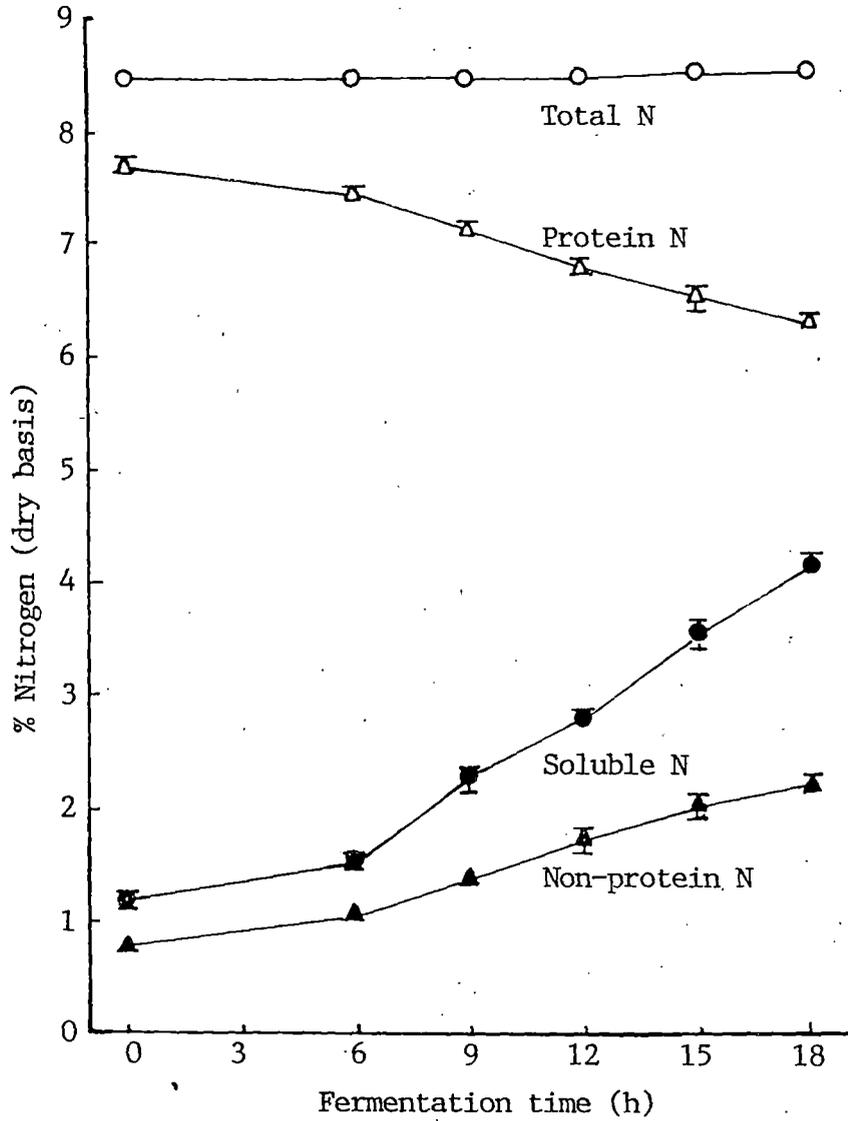


Fig. 45. Changes in nitrogen content in soya beans during monoculture fermentation with *Bacillus subtilis* DK-W1 for kinema production at 45°C. Values are the means of three batches of fermentation with ranges.

Table 28. Proximate composition of raw ingredient and sinki from different sources

Parameters	Radish tap root	Sinki			
		Dry			Fresh
		Darjeeling market	Kalimpong market	Gangtok market	Laboratory-made
Moisture (%)	95.4 (94.8-95.9)	22.0 (20.0-24.0)	20.4 (18.6-22.5)	21.3 (19.0-23.2)	93.5 (93.4-93.7)
Protein (% DM) (Total N x 6.25)	14.6 (14.1-14.7)	14.5 (14.0-14.7)	14.5 (14.0-14.6)	14.6 (13.9-14.8)	14.6 (14.1-14.7)
Fat (% DM) (Ether extract)	2.5 (2.0-3.0)	2.5 (2.0-3.2)	2.5 (2.0-3.1)	2.5 (2.0-3.0)	2.5 (2.0-3.1)
Ash (% DM)	11.5 (11.3-12.5)	11.3 (10.6-12.5)	11.4 (10.8-12.1)	11.5 (11.0-12.5)	11.3 (10.8-12.3)
pH	6.72 (6.60-6.80)	4.45 (4.00-4.80)	4.28 (3.90-4.55)	4.40 (4.00-4.68)	3.30 (3.25-3.35)
Titratable acidity (as % lactic acid)	0.04 (0.02-0.05)	0.65 (0.55-0.80)	0.80 (0.70-0.95)	0.72 (0.60-0.85)	1.28 (1.15-1.40)

% DM, percentage on dry matter basis

Data represent the means of 10 samples. Ranges are given in parentheses.

moisture content of radish tap root was 95.4% which was slightly reduced to 93.5% in freshly prepared sinki as revealed in the laboratory. The sun-dried market sinki had the mean moisture content of only 21.3%. The content of protein, fat and ash was relatively same in the substrate and the fermented product. The mean pH value of tap root was 6.72, whereas that of freshly prepared sinki was 3.30. But, the sun-dried market sinki had the mean pH of 4.38. Mean titratable acidity increased from 0.04% in radish tap root to 1.28% in fresh laboratory-made sinki. But, the sun-dried market sinki had 0.72% acidity.

4.3.2. Microbial analysis

4.3.2.1. Isolation of microorganisms from market and laboratory-made samples

Pure culture colonies developed well on MRS agar, GYP-CaCO₃ agar and APT agar, and poorly on nutrient agar, whereas there was no colony on YM agar and PDA supplemented with penicillin and streptomycin. A total of 453 strains of bacteria, isolated from 40 samples of sinki, were grouped on the basis of gas from glucose, ammonia from arginine, growth at 15°C and acid from a host of sugars (Table 29). Twelve representative strains, one from each group, were selected randomly for identification.

4.3.2.2. Taxonomical studies

All the isolates formed small (1-5 mm), convex, smooth colonies with entire margin and without pigment on MRS agar. All of them were regular, non-sporing, Gram positive rods. They were

Table 29. Selection of representative strains of bacteria isolated from sinki samples^a

Source	Number of strains ^b isolated	Gas from glucose	NH ₃ from arginine	Growth at 15°C	Acid produced from ^c						Grouped strains	Representative strains		
					Ara	Xyl	Tre	Manl	Sorl	Cel			Lac	
Darjeeling market	127	-	-	+	+	-	+	+	+	+	+	52	DS-R1	
		-	-	+	-	+	+	+	+	+	+	30	DS-R7	
		+	+	+	+	-	-	-	-	-	+	45	DS-SR1	
Kalimpong market	98	-	-	+	+	+	+	+	+	+	+	55	KS-R1	
		+	+	+	+	+	-	-	-	-	-	43	KS-SR1	
Gangtok market	108	-	-	+	-	-	+	+	+	+	+	68	GS-R1	
		+	+	+	+	+	-	-	-	-	+	40	GS-SR1	
Laboratory-made	120	-	-	+	-	+	+	+	+	+	+	53	LS-R1	
		-	-	-	+	+	+	+	+	+	+	19	LS-R9	
		+	+	+	+	-	-	-	-	-	-	27	LS-SR1	
		+	+	-	+	-	+	-	-	-	+	+	13	LS-MS1
		+	+	-	-	+	-	-	-	-	+	+	8	LS-MS2

^aNumber of samples was 10 from each source.

^bAll the isolates were non-motile, non-sporeforming, Gram positive and catalase negative rods.

^cAra, L-arabinose; Xyl, D-xylose; Tre, trehalose; Manl, mannitol; Sorl, sorbitol; Cel, cellobiose; Lac, lactose

microaerophilic and catalase negative. According to the criteria laid down by Kandler and Weiss (1986), these isolates belonged to the genus *Lactobacillus*. Their detailed morphological and physiological characteristics are shown in Table 30. The strains DS-R1, DS-R7, KS-R1, GS-R1 (Fig. 46), LS-R1 and LS-R9 were identified as *Lactobacillus plantarum* Orla-Jensen; the strains DS-SR1, KS-SR1, GS-SR1 and LS-SR1 (Fig. 47) as *Lactobacillus brevis* Orla-Jensen; and the strains LS-MS1 (Fig. 48) and LS-MS2 were identified as *Lactobacillus fermentum* Beijerinck.

4.3.2.3. Microbial load in substrate and products

The average value of microbial load studied in 10 samples of sinki collected from each of Darjeeling, Kalimpong and Gangtok markets, and of laboratory-made as well as radish (*Raphanus sativus* L.) tap root are presented in Table 31 and Fig. 49. The substrate showed the presence of three species of *Lactobacillus* including *L. plantarum*, *L. brevis* and *L. fermentum*. In sinki, *L. plantarum* was the most dominant bacterium (10^8 - 10^9 cfu/g) followed by *L. brevis* (10^3 - 10^4 cfu/g). Interestingly, while *L. fermentum* was the predominant bacterial type in the substrate, it was absent in the fermented product.

Table 32 shows the prevalence of microorganisms in substrate and products. All the three species of *Lactobacillus* occurred in 100% of the radish tap root samples. In both market and laboratory-made sinki, the prevalence of *L. plantarum* and *L. brevis* was 100%.

Table 30. Characteristics of representative strains of lactic acid bacteria isolated from sinki samples

Parameters	DS-R1	DS-R7	KS-R1	GS-R1	LS-R1	LS-R9	DS-SR1	KS-SR1	GS-SR1	LS-SR1	LS-MS1	LS-MS2
Cell morphology												
Shape	Rod ^a	Rod										
Size												
Width (µm)	0.7-1.0	0.7-1.1	0.5-1.0	0.7-1.1	0.7-1.0	0.5-1.0	0.6-0.7	0.6-0.7	0.6-0.7	0.6-0.7	0.6-0.8	0.5-0.8
Length (µm)	2.0-5.0	2.0-5.0	1.5-4.5	2.0-5.0	2.0-5.0	2.0-5.0	2.0-3.0	2.0-3.0	2.0-3.5	2.0-3.5	1.5-3.0	1.5-3.0
Spore	-	-	-	-	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-	-	-	-	-
Gram reaction	+	+	+	+	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-	-	-	-	-
O ₂ requirement	F ^b	F	F	F	F	F	F	F	F	F	F	F
Gas from glucose	-	-	-	-	-	-	+	+	+	+	+	+
Hydrolysis of												
Fat	-	-	-	-	-	-	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-	-	-	-	-	-	-
Arginine	-	-	-	-	-	-	+	+	+	+	+	+
Esculin	+	+	+	+	+	+	+	+	-	+	-	-

Parameters	DS-R1	DS-R7	KS-R1	GS-R1	LS-R1	LS-R9	DS-SR1	KS-SR1	GS-SR1	LS-SR1	LS-MS1	LS-MS2
Growth at												
15°C	+	+	+	+	+	+	+	+	+	+	-	-
45°C	+	-	+	+	+	-	-	-	+	+	+	+
Optimum temperature for growth (°C)	30	30	30	30	30	30	30	30	30	30	30	30
Reduction of												
nitrate to nitrite	-	-	-	-	-	-	-	-	-	-	-	-
Reduction of												
nitrite to N ₂	-	-	-	-	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-	-	-	-	-
Acid produced from												
L-Arabinose	+	-	+	-	-	+	+	+	+	+	+	-
D-Xylose	-	+	+	-	+	+	-	+	+	-	-	+
D-Ribose	+	+	+	+	+	+	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	+	+	-	+	+
D-Mannose	+	+	+	+	+	+	-	-	-	-	+	+

Parameters	DS-R1	DS-R7	KS-R1	GS-R1	LS-R1	LS-R9	DS-SR1	KS-SR1	GS-SR1	LS-SR1	LS-MS1	LS-MS2
Maltose	+	+	+	+	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	-	+	-	+	+
Sucrose	+	+	+	+	+	+	-	-	+	+	+	+
Cellobiose	+	+	+	+	+	+	-	-	-	-	+	+
Trehalose	+	+	+	+	+	+	-	-	-	-	+	-
Melibiose	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	+	-	-	+	+	-	+	-	+	-	+	-
Mannitol	+	+	+	+	+	+	-	-	-	-	-	-
Sorbitol	+	+	+	+	+	+	-	-	-	-	-	-
Starch	-	-	-	-	-	-	-	-	-	-	-	-

^aIn chain of 2-4 cells

^bFacultative anaerobe

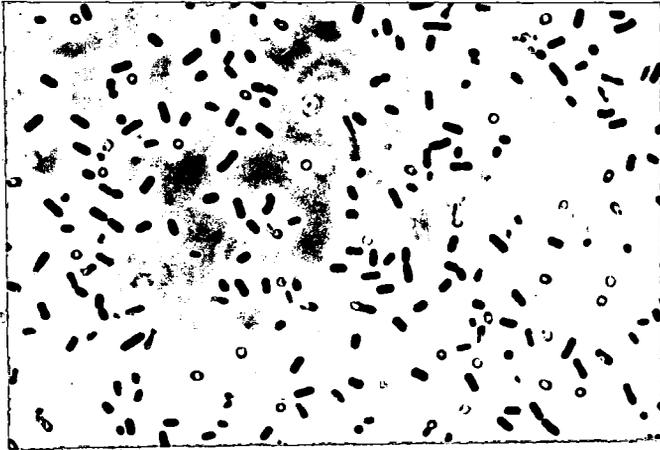


Fig. 46. *Lactobacillus plantarum* GS-R1 (APT agar, 3 days, 30°C) isolated from sinki; phase contrast micrograph (x 1120)

Fig. 47. *Lactobacillus brevis* LS-SR1 (APT agar, 3 days, 30°C) isolated from sinki; phase contrast micrograph (x 1120)



Fig. 48. *Lactobacillus fermentum* LS-MS1 (APT agar, 3 days, 30°C) isolated from fermenting radish tap root during sinki preparation; phase contrast micrograph (x 1120)

Table 31. Microbial load of raw ingredient and sinki from different sources

Microorganisms	Radish tap root	cfu(x 10 ⁶)/g fresh weight			
		Sinki			
		Darjeeling market	Kalimpong market	Gangtok market	Laboratory-made
<i>Lactobacillus plantarum</i>	0.1 (0.007-0.2)	625.0 (460.0-775.0)	646.0 (490.0-810.0)	637.0 (472.0-790.0)	650.0 (495.0-800.0)
<i>Lactobacillus brevis</i>	0.1 (0.008-0.2)	0.006 (0.0003-0.009)	0.007 (0.0003-0.01)	0.006 (0.004-0.009)	0.007 (0.0003-0.01)
<i>Lactobacillus fermentum</i>	0.5 (0.04-0.8)	0	0	0	0

cfu, colony forming units

Data represent the means of 10 samples. Ranges are given in parentheses.

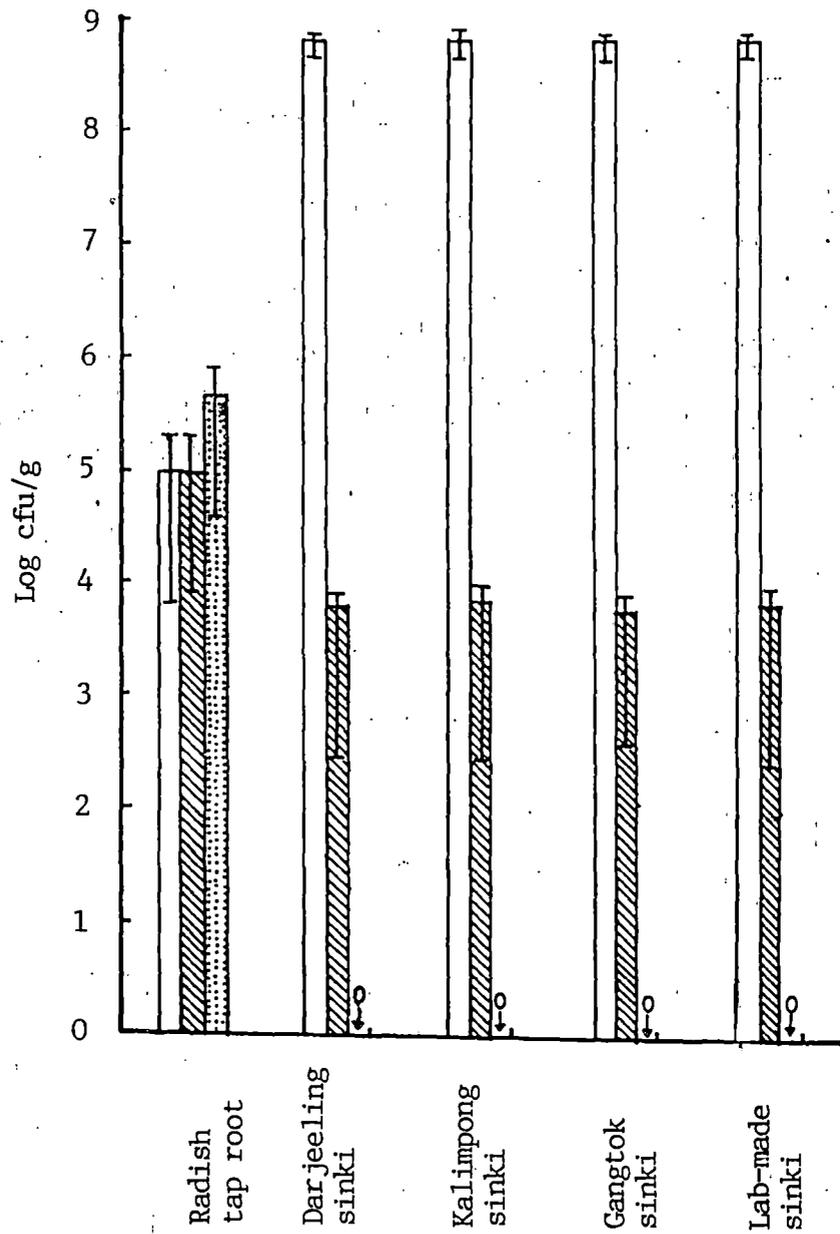


Fig. 49. Microbial load of raw ingredient and sinki from different sources. Values are the means with ranges of 10 samples. *Lactobacillus plantarum*, ; *Lactobacillus brevis*, ; *Lactobacillus fermentum*, 

Table 32. Prevalence of various microorganisms in raw ingredient and sinki from different sources

Microorganisms	Positive samples (%) ^a				
	Radish tap root	Sinki			
		Darjeeling market	Kalimpong market	Gangtok market	Laboratory-made
<i>Lactobacillus plantarum</i>	100	100	100	100	100
<i>Lactobacillus brevis</i>	100	100	100	100	100
<i>Lactobacillus fermentum</i>	100	0	0	0	0

^aExpressed on the basis of a total of 10 samples studied as in Table 31

4.3.3. Optimization of traditional process parameters

Table 33 represents the average sensory scores for optimizing fermentation container of radish tap root for sinki preparation. All the treatments differed significantly ($P < 0.05$) among themselves with respect to taste, flavour and total score. However, in respect of colour, there was no significant difference ($P < 0.05$) between treatments A and B. The treatment B, glass jar with lid as a fermenting container, had the highest score.

All the treatments considered for optimizing temperature of incubation during fermentation differed significantly ($P < 0.05$) from each other in respect of every sensory attribute (Table 34). The treatment B, temperature of incubation at 30°C during fermentation, had the highest score.

All the treatments considered for optimizing fermentation time differed significantly ($P < 0.05$) from each other in respect of taste, flavour and total score (Table 35). However, in respect of colour, the treatments B and C had no significant difference ($P < 0.05$) between themselves. The treatment B, fermentation time of 12 days, had the highest score.

4.3.4. Successional studies on radish tap root during sinki production

Sinki was prepared in the laboratory under optimized conditions for studies on microbial and biochemical changes. Radish (*Raphanus sativus* L.) tap root was washed, wilted (2 days), sliced, again washed and squeezed to drain excess water. The slices were placed in glass jar, pressed tightly, covered with lid and fermented at

Table 33. Average sensory scores for optimizing fermentation container of radish tap root for sinki production

Attributes	Fermentation container		
	A Earthen jar	B Glass jar with lid	C Polythene bag
Taste	49.7 ^b (49.5-50.0)	53.3 ^a (53.0-53.5)	44.8 ^c (44.2-45.3)
Flavour	29.0 ^b (28.8-29.3)	32.3 ^a (31.8-32.8)	24.0 ^c (23.8-24.3)
Colour	4.0 ^a (4.0-4.0)	4.5 ^a (4.4-4.5)	2.3 ^b (2.0-3.0)
Total score	82.7 ^b (82.3-83.3)	90.1 ^a (89.2-90.8)	71.1 ^c (70.0-72.6)

Temperature of incubation, 30°C; fermentation time, 12 days

Data represent the means of three replications. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly ($P < 0.05$).

Table 34. Average sensory scores for optimizing temperature of incubation during fermentation of radish tap root for sinki production

Attributes	Temperature of incubation (°C)		
	A 20	B 30	C 40
Taste	49.9 ^b (49.8-50.0)	53.2 ^a (52.5-54.0)	46.0 ^c (45.8-46.3)
Flavour	26.8 ^b (26.5-27.0)	32.3 ^a (31.5-32.8)	25.3 ^c (25.0-26.0)
Colour	3.9 ^b (3.8-4.0)	4.5 ^a (4.0-5.0)	3.0 ^c (2.8-3.3)
Total score	80.6 ^b (80.1-81.0)	90.0 ^a (88.0-91.8)	74.3 ^c (73.6-75.6)

Fermentation container, glass jar with lid; fermentation time, 12 days

Data represent the means of three replications. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly ($P < 0.05$).

Table 35. Average sensory scores for optimizing fermentation time during fermentation of radish tap root for sinki production

Attributes	Fermentation time (days)		
	A 7	B 12	C 20
Taste	43.7 ^c (43.6-43.8)	53.6 ^a (53.5-53.6)	50.7 ^b (50.7-50.8)
Flavour	25.0 ^c (24.8-25.3)	32.2 ^a (32.0-32.5)	29.7 ^b (29.5-29.8)
Colour	3.0 ^b (3.0-3.0)	4.4 ^a (4.3-4.5)	4.4 ^a (4.3-4.5)
Total score	71.7 ^c (71.4-72.1)	90.2 ^a (89.8-90.6)	84.8 ^b (84.4-85.1)

Fermentation container, glass jar with lid; temperature of incubation, 30°C.

Data represent the means of three replications. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly ($P < 0.05$).

30°C for 12 days. The successional studies were carried out at every two days intervals within a range of 0-12 days.

4.3.4.1. Microbial changes

Table 36 and Fig. 50 show the changes in microflora in radish tap root during sinki production. During the first 2 days of fermentation, *Lactobacillus fermentum* predominated. At that time, its population size increased by 50 fold. The 2nd day onward, its number decreased sharply; since the 4th day, the decrease was significant ($P < 0.05$) at every 2 days intervals, and at the end of fermentation it disappeared from the product. The population of *L. brevis* increased significantly ($P < 0.05$) from 10^5 cfu/g at the start to 10^7 cfu/g at 4 days, after which its number decreased significantly ($P < 0.05$) at every 2 days intervals to 10^3 cfu/g at the end. On the other hand, compared to the others, the population size of *L. plantarum* was minimum at the onset of fermentation, but its load increased significantly ($P < 0.05$) at 2 days intervals till the first 6 days of fermentation. The 6th day onward, *L. plantarum* formed the major component of the microflora of the fermenting mass.

4.3.4.2. Biochemical changes

Biochemical changes in radish tap root during sinki production are presented in Table 37. The mean pH value decreased from 6.72 at the start to 3.30 at the end of fermentation (Fig. 51). The titratable acidity increased significantly ($P < 0.05$) at every 2 days intervals till the 10th day, after which there was no significant rise ($P < 0.05$) in acidity (Fig. 51). The moisture and

Table 36. Changes in microflora in radish tap roots during sinki production

Fermentation time (days)	cfu (x 10 ⁶)/g fresh weight		
	<i>Lactobacillus fermentum</i>	<i>Lactobacillus brevis</i>	<i>Lactobacillus plantarum</i>
0	0.4 ^a (0.04-0.7)	0.1 ^a (0.007-0.2)	0.07 ^a (0.004-0.1)
2	20.0 ^b (12.0-25.0)	8.0 ^b (6.0-9.0)	1.0 ^b (0.5-2.0)
4	5.2 ^b (2.7-7.1)	23.0 ^c (19.0-27.5)	10.0 ^c (8.0-14.1)
6	0.2 ^a (0.008-0.3)	5.0 ^b (1.0-7.2)	130.0 ^d (70.0-190.0)
8	0.001 ^c (0.0002-0.002)	0.2 ^a (0.07-0.4)	355.0 ^{de} (195.0-520.0)
10	0.00002 ^d (0-0.00004)	0.02 ^d (0.008-0.03)	580.0 ^e (370.0-705.0)
12	0	0.004 ^e (0.0008-0.006)	670.0 ^e (490.0-825.0)

cfu, colony forming units

Data represent the means of five batches of fermentation. Ranges are given in parentheses.

Data were transformed into logarithmic values and then subjected to analysis of variance. Values bearing different superscripts in each column differ significantly ($P < 0.05$).

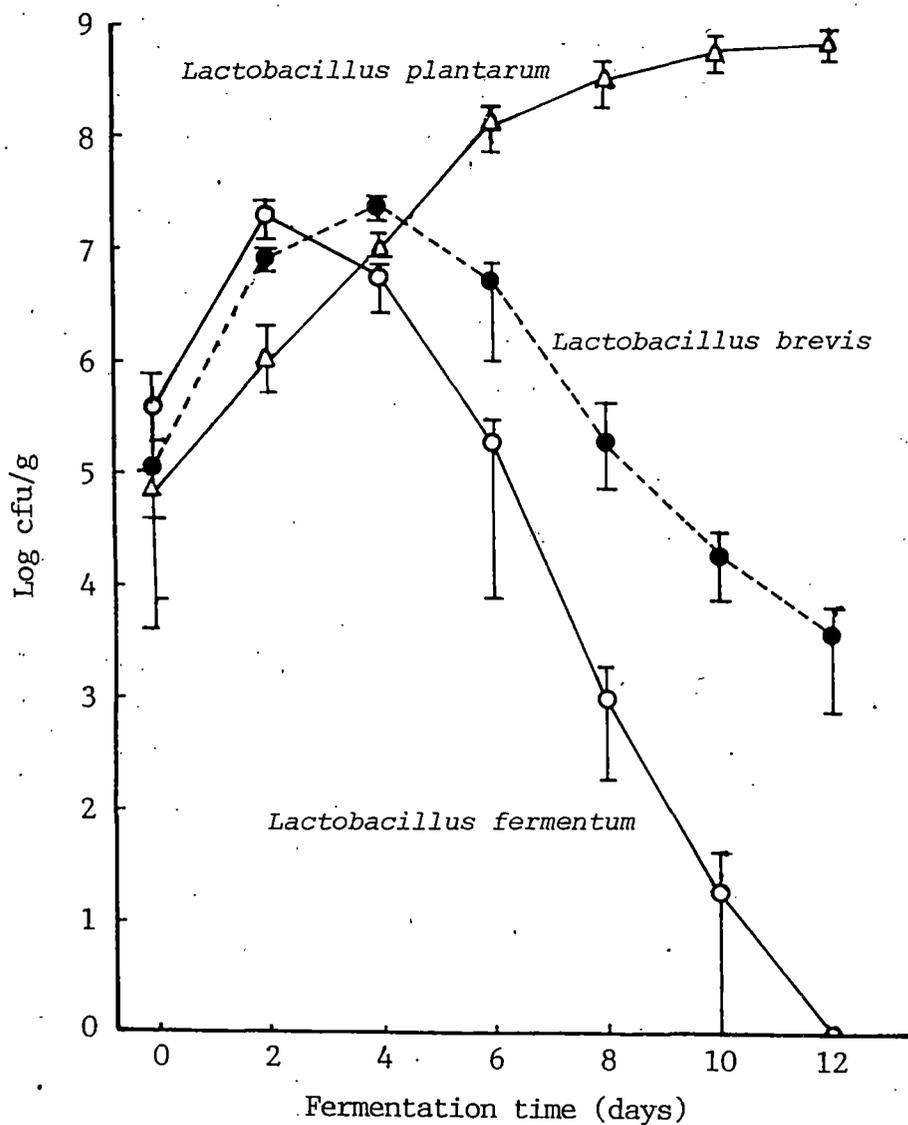


Fig. 50. Changes in microflora in radish tap roots during sinki production. Values are the means of five batches of fermentation and bars represent ranges.

Table 37. Biochemical changes in radish tap roots during sinki production

Fermentation time (days)	pH	Titrateable acidity (as % lactic acid)	Moisture (%)	Total nitrogen (% DM)
0	6.72 ^a (6.60-6.80)	0.04 ^a (0.03-0.06)	93.66 ^a (93.61-93.71)	2.34 ^a (2.32-2.36)
2	5.18 ^b (4.90-5.50)	0.38 ^b (0.32-0.42)	93.66 ^a (93.61-93.71)	2.34 ^a (2.32-2.36)
4	4.15 ^c (3.95-4.41)	0.58 ^c (0.50-0.67)	93.66 ^a (93.60-93.70)	2.34 ^a (2.31-2.36)
6	3.82 ^d (3.70-3.95)	0.65 ^d (0.60-0.72)	93.65 ^a (93.60-93.70)	2.33 ^a (2.31-2.36)
8	3.50 ^e (3.40-3.60)	0.83 ^e (0.75-0.95)	93.65 ^a (93.60-93.70)	2.33 ^a (2.31-2.36)
10	3.35 ^{ef} (3.30-3.40)	1.23 ^f (1.15-1.35)	93.65 ^a (93.60-93.70)	2.33 ^a (2.31-2.36)
12	3.30 ^f (3.25-3.35)	1.28 ^f (1.15-1.40)	93.64 ^a (93.60-93.70)	2.33 ^a (2.30-2.36)

% DM, percentage on dry matter basis

Data represent the means of five batches of fermentation. Ranges are given in parentheses.

Values bearing different superscripts in each column differ significantly ($P < 0.05$).

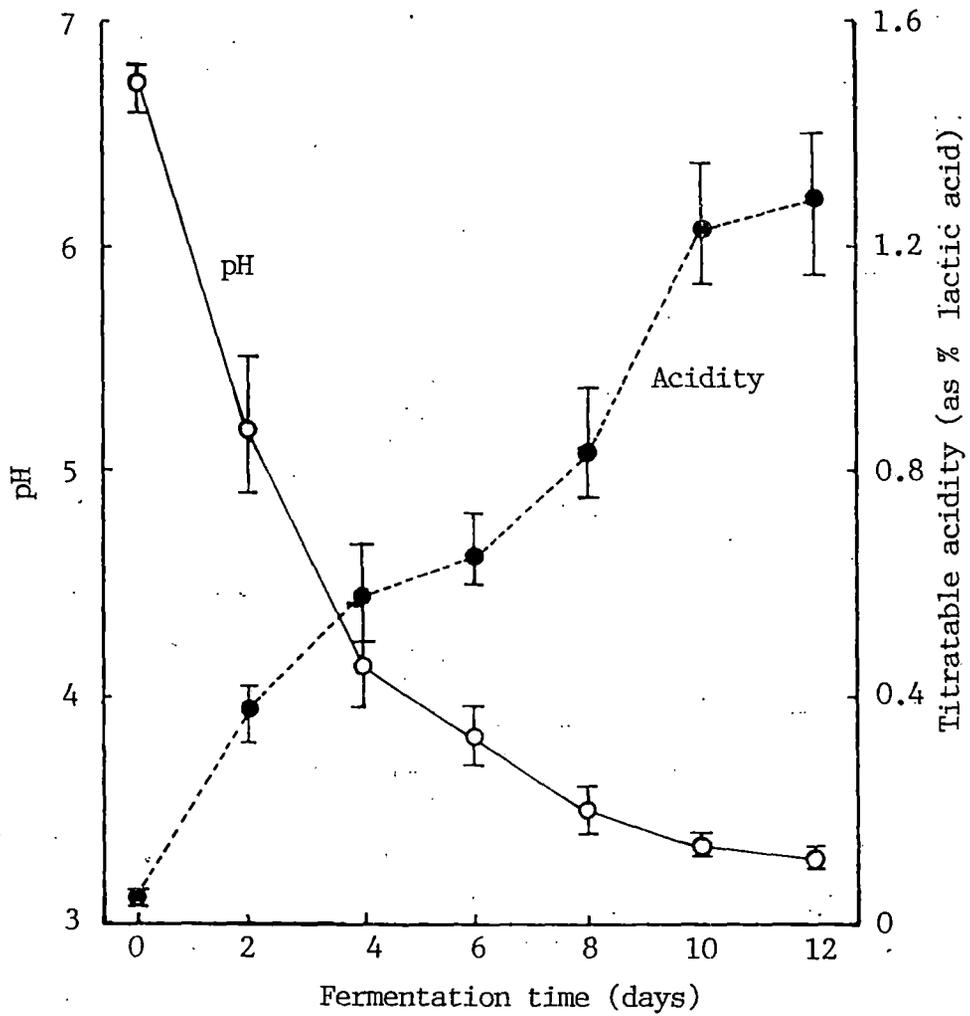


Fig. 51. Changes in pH and titratable acidity in radish tap roots for sinki production. Values are the means of five batches of fermentation with ranges. Where ranges overlap, bars for the lowest mean are shown to the left.

and total nitrogen contents remained relatively constant throughout fermentation.

4.4. MESU

4.4.1. Proximate composition

The proximate composition of young bamboo shoot and mesu from different sources is presented in Table 38. The contents of moisture, protein, fat and ash of mesu were relatively as those of bamboo shoot. The mean pH value of the substrate was 6.35, whereas that of mesu was 4.04. The mean titratable acidity in unfermented bamboo shoot was only 0.04%, while the same in the fermented product was 0.83%.

4.4.2. Microbial analysis

4.4.2.1. Isolation of microorganisms from market and laboratory-made samples

Pure culture colonies developed on MRS agar, GYP-CaCO₃ agar and APT agar, and poorly on nutrient agar, whereas there was no colony on YM agar and PDA supplemented with penicillin and streptomycin. A total of 327 strains of bacteria, isolated from 30 samples of mesu, were grouped on the basis of cell shape, gas from glucose, ammonia from arginine, and acid from a host of sugars (Table 39). Twelve representative strains, one from each group, were selected randomly for identification.

Table 38. Proximate composition of raw ingredient and mesu from different sources

Parameters	Bamboo shoot	Mesu		
		Kalimpong market	Gangtok market	Laboratory-made
Moisture (%)	94.2 (93.9-94.4)	94.2 (93.9-94.3)	94.1 (93.9-94.2)	94.1 (94.0-94.2)
Protein (% DM) (Total N x 6.25)	17.4 (17.1-17.7)	17.1 (16.9-17.6)	17.1 (16.5-17.6)	17.2 (16.9-17.6)
Fat (% DM) (Ether extract)	2.4 (2.2-2.5)	2.4 (2.3-2.5)	2.4 (2.2-2.4)	2.4 (2.3-2.5)
Ash (% DM)	11.2 (10.6-11.8)	11.0 (10.5-11.7)	10.9 (10.4-11.8)	10.9 (10.4-11.7)
pH	6.35 (6.16-6.50)	4.08 (3.80-4.44)	4.20 (3.95-4.60)	3.84 (3.68-3.95)
Titratable acidity (as % lactic acid)	0.04 (0.03-0.05)	0.80 (0.63-0.91)	0.75 (0.56-0.90)	0.95 (0.90-1.00)

% DM, percentage on dry matter basis

Data represent the means of 10 samples. Ranges are given in parentheses.

Table 39. Selection of representative strains of bacteria isolated from mesu samples^a

Source	Number of strains ^b isolated	Cell shape	Gas from glucose	NH ₃ from arginine	Acid produced from ^c							Grouped strains	Representative strains
					Ara	Xyl	Tre	Manl	Sorl	Cel	Lac		
Kalimpong market	109	Rod	-	-	+	-	+	+	+	+	+	52	KM-R1
		Rod	-	-	-	-	+	+	+	+	+	23	KM-R32
		Rod	+	+	+	-	-	-	-	-	-	19	KM-SR1
		Coccus	-	+	+	-	+	-	-	+	-	15	KM-T1
Gangtok market	103	Rod	-	-	+	+	+	+	+	+	+	49	GM-R1
		Rod	+	+	+	+	-	-	-	-	+	36	GM-SR1
		Coccus	-	+	+	+	+	-	-	+	+	18	GM-T1
Laboratory-made	115	Rod	-	-	-	-	+	+	+	+	+	38	LM-R1
		Rod	-	-	+	+	+	+	+	+	+	20	LM-R14
		Rod	+	+	+	-	-	-	-	-	+	30	LM-SR1
		Coccus	-	+	+	-	+	-	-	+	+	13	LM-T1
		Coccus	-	+	+	+	+	+	-	-	+	-	14

^aNumber of samples was 10 from each source.

^bAll the isolates were non-motile, non-sporeforming, Gram positive and catalase negative.

^cAra, L-arabinose; Xyl, D-xylose; Tre, trehalose; Manl, mannitol; Sorl, sorbitol; Cel, cellobiose; Lac, lactose

4.4.2.2. Taxonomical studies

All the isolates were non-motile, non-sporing, microaerophilic, catalase negative and Gram positive. The rod-shaped cells (strains KM-R1, KM-R32, KM-SR1, GM-R1, GM-SR1, LM-R1, LM-R14 and LM-SR1) were assigned to the genus *Lactobacillus*, whereas the spherical cells in tetrads (strains KM-T1, GM-T1, LM-T1 and LM-T2) belonged to the genus *Pediococcus*, according to the criteria laid down by Kandler and Weiss (1986) and Garvie (1986b), respectively. Their detailed characteristics are shown in Tables 40 and 41. While the strains KM-R1, KM-R32, GM-R1 (Fig. 52), LM-R1 and LM-R14 were identified as *Lactobacillus plantarum* Orla-Jensen, the strains KM-SR1, GM-SR1 and LM-SR1 (Fig. 53) were identified as *Lactobacillus brevis* Orla-Jensen. The tetrad strains KM-T1, GM-T1, LM-T1 (Fig. 54) and LM-T2 were identified as *Pediococcus pentosaceus* Mess.

4.4.2.3. Microbial load in substrate and products

The average value of microbial load studied in 10 samples of mesu collected from each of Kalimpong and Gangtok markets and laboratory-made as well as young bamboo shoot (*Dendrocalamus hamiltonii* Nees.) are presented in Table 42 and Fig. 55. All the three species including *Lactobacillus plantarum*, *L. brevis* and *Pediococcus pentosaceus* were present in the substrate as well as the fermented products. In the substrate, *P. pentosaceus* was the most predominant species, whereas in mesu, *L. plantarum* was most abundant and *P. pentosaceus* was least populated.

Table 43 shows the prevalence of microorganisms in the substrate and the products. All the three microorganisms including

Table 40. Characteristics of representative strains of asporogenous rod-shaped bacteria isolated from mesu samples

Parameters	KM-R1	KM-R32	GM-R1	LM-R1	LM-R14	KM-SR1	GM-SR1	LM-SR1
Cell morphology								
Shape	Rod ^a	Rod						
Size								
Width (μm)	$\emptyset.7\text{-}\emptyset.8$	$\emptyset.7\text{-}\emptyset.8$	$\emptyset.7\text{-}\emptyset.9$	$\emptyset.7\text{-}\emptyset.8$	$\emptyset.7\text{-}\emptyset.9$	$\emptyset.6\text{-}\emptyset.7$	$\emptyset.6\text{-}\emptyset.7$	$\emptyset.6\text{-}\emptyset.7$
Length (μm)	$2.0\text{-}4.5$	$2.0\text{-}4.7$	$2.0\text{-}5.0$	$1.5\text{-}4.5$	$2.0\text{-}5.0$	$2.0\text{-}3.0$	$2.0\text{-}3.5$	$2.0\text{-}3.0$
Spore	-	-	-	-	-	-	-	-
Motility	-	-	-	-	-	-	-	-
Gram reaction	+	+	+	+	+	+	+	+
Catalase	-	-	-	-	-	-	-	-
O ₂ requirement	F ^b	F	F	F	F	F	F	F
Gas from glucose	-	-	-	-	-	+	+	+
Hydrolysis of								
Fat	-	-	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-	-	-
Arginine	-	-	-	-	-	+	+	+
Esculin	+	+	+	+	+	+	+	-

Parameters	KM-R1	KM-R32	GM-R1	LM-R1	LM-R14	KM-SR1	GM-SR1	LM-SR1
Growth at								
15°C	+	+	+	+	+	+	+	+
45°C	-	+	+	+	-	-	-	+
Optimum temperature								
for growth (°C)	30	30	30	30	30	30	30	30
Reduction of nitrate to nitrite	-	-	-	-	-	-	-	-
Reduction of nitrite to N ₂	-	-	-	-	-	-	-	-
Indole production	-	-	-	-	-	-	-	-
Acid produced from								
L-Arabinose	+	-	+	-	+	+	+	+
D-Ribose	+	+	+	+	+	+	+	+
D-Xylose	-	-	+	-	+	-	+	-
D-Glucose	+	+	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	+	-
D-Mannose	-	-	-	-	-	-	-	-
Maltose	+	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	-	+	+

Parameters	KM-R1	KM-R32	GM-R1	LM-R1	LM-R14	KM-SR1	GM-SR1	LM-SR1
Cellobiose	+	+	+	+	+	-	-	-
Sucrose	+	+	+	+	+	+	-	-
Melibiose	+	+	+	+	+	+	+	+
Trehalose	+	+	+	+	+	-	-	-
Raffinose	+	-	-	-	+	-	+	-
Mannitol	+	+	+	+	+	-	-	-
Sorbitol	+	+	+	+	+	-	-	-
Starch	-	-	-	-	-	-	-	-

^aIn chain of 2-4 cells

^bFacultative anaerobe

Table 41. Characteristics of representative strains of tetrad bacteria isolated from mesu samples

Parameters	KM-T1	GM-T1	LM-T1	LM-T2
Cell morphology				
Shape	Coccus	Coccus	Coccus	Coccus
Arrangement	Tetrad	Tetrad	Tetrad	Tetrad
Size: diameter (μm)	$\emptyset.6-\emptyset.7$	$\emptyset.6-\emptyset.7$	$\emptyset.6-\emptyset.7$	$\emptyset.6-\emptyset.7$
Spore	-	-	-	-
Motility	-	-	-	-
Gram reaction	+	+	+	+
Catalase	-	-	-	-
O ₂ requirement	F ^a	F	F	F
Gas from glucose	-	-	-	-
Hydrolysis of				
Fat	-	-	-	-
Gelatin	-	-	-	-
Arginine	+	+	+	+
Esculin	+	+	+	+
Growth in NaCl				
4.0%	+	+	+	+
6.5%	+	+	+	+
18.0%	-	-	-	-
Growth in pH 4.2-8.5	+	+	+	+
Growth at 37-45°C	+	+	+	+
Growth at 50°C	-	-	-	-
Optimum temperature for growth (°C)	30	30	30	30
Reduction of nitrate to nitrite	-	-	-	-
Reduction of nitrite to N ₂	-	-	-	-

Parameters	KM-T1	GM-T1	LM-T1	LM-T2
Indole production	-	-	-	-
Acid produced from				
L-Arabinose	+	+	+	+
Cellobiose	+	+	+	+
Fructose	+	+	+	+
D-Glucose	+	+	+	+
D-Galactose	+	+	+	+
Lactose	-	+	+	-
Maltose	+	+	+	+
Mannitol	-	-	-	-
Mannose	-	-	-	-
Melibiose	+	+	+	+
Raffinose	-	-	-	-
D-Ribose	+	+	+	+
Sorbitol	-	-	-	-
Sucrose	-	-	-	-
Starch	-	-	-	-
Trehalose	+	+	+	+
Xylose	-	+	-	+

^aFacultative anaerobe:



Fig. 52. *Lactobacillus plantarum* GM-R1
(APT agar, 3 days, 30°C) isolated from
mesu; phase contrast micrograph (x
1120)

Fig. 53. *Lactobacillus brevis* LM-SR1.
(APT agar, 3 days, 30°C) isolated from
mesu; phase contrast micrograph (x
1120)

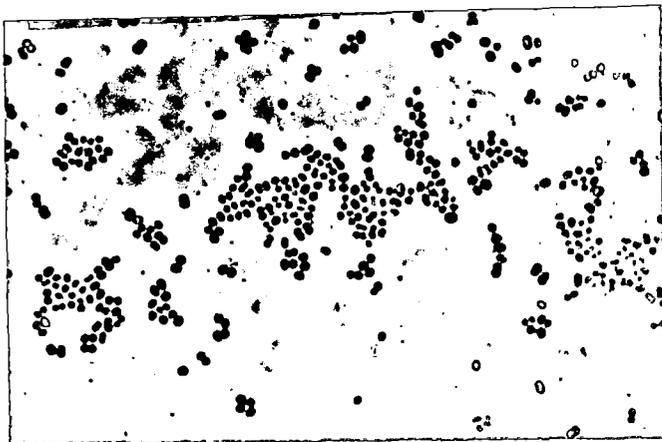
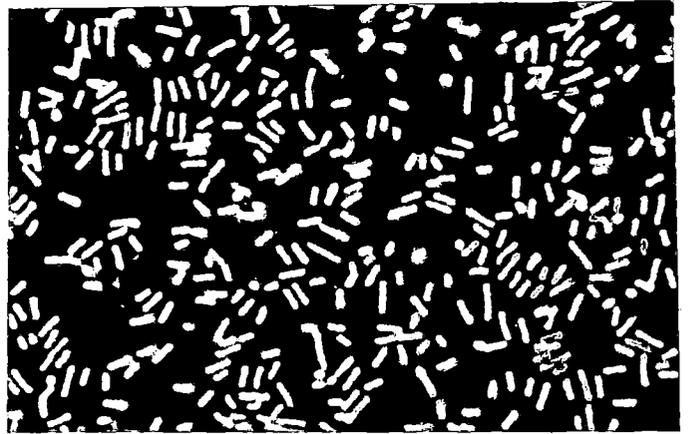


Fig. 54. *Pediococcus pentosaceus* LM-T1
(APT agar, 3 days, 30°C) isolated from
mesu, showing coccal cells in tetrads;
phase contrast micrograph (x 1120)

Table 42. Microbial load of raw ingredient and mesu from different sources

Microorganisms	cfu(x 10 ⁶)/g fresh weight			
	Bamboo shoot	Mesu		
		Kalimpong market	Gangtok market	Laboratory-made
<i>Lactobacillus plantarum</i>	0.1 (0.006-0.2)	283.0 (227.0-331.0)	290.0 (234.0-395.0)	300.0 (240.0-390.0)
<i>Lactobacillus brevis</i>	0.08 (0.005-0.2)	0.004 (0.0008-0.008)	0.005 (0.0008-0.008)	0.004 (0.0008-0.009)
<i>Pediococcus pentosaceus</i>	0.2 (0.007-0.4)	0.00002 (0-0.00004)	0.00003 (0-0.00006)	0.00002 (0-0.00004)

cfu, colony forming units

Data represent the means of 10 samples. Ranges are given in parentheses.

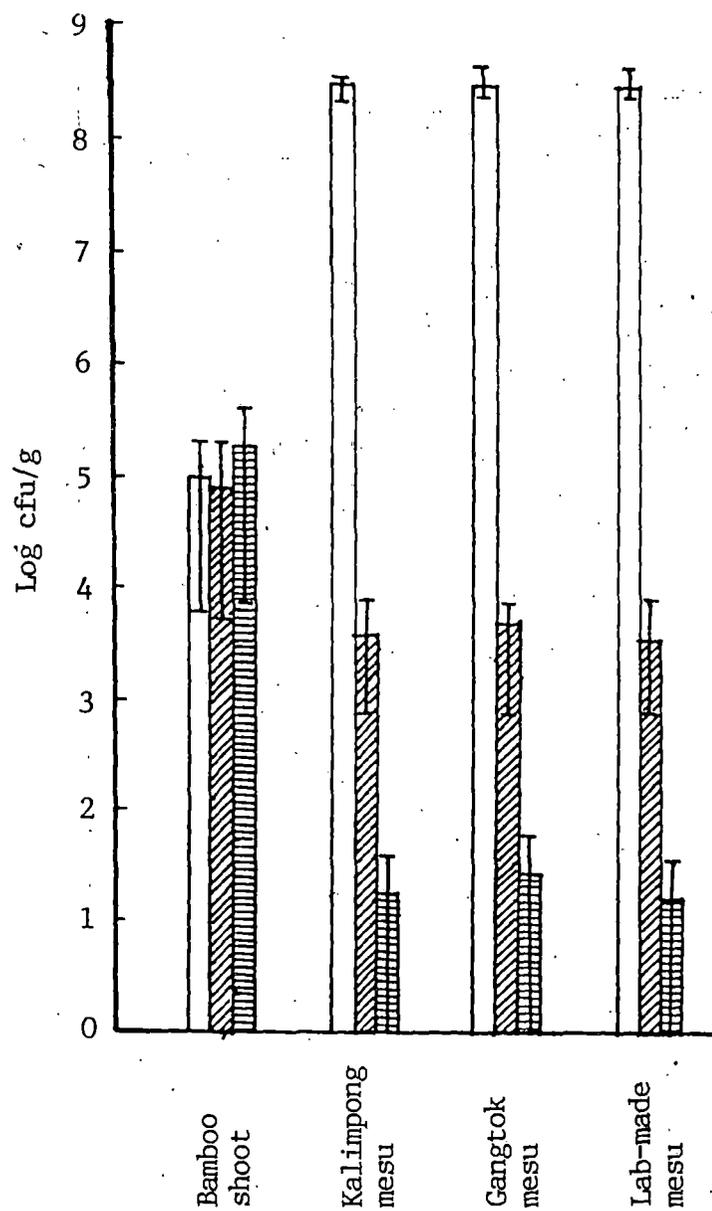


Fig 55. Microbial load of raw ingredient and mesu from different sources. Values are the means with ranges of 10 samples. *Lactobacillus plantarum*, ; *Lactobacillus brevis*, ; *Pediococcus pentosaceus*, 

Table 43. Prevalence of various microorganisms in raw ingredient and mesu from different sources

Microorganisms	Positive samples (%) ^a			
	Bamboo shoot	Mesu		
		Kalimpong market	Gangtok market	Laboratory-made
<i>Lactobacillus plantarum</i>	100	100	100	100
<i>Lactobacillus brevis</i>	100	100	100	100
<i>Pediococcus pentosaceus</i>	100	50	40	50

^aExpressed on the basis of a total of 10 samples studied as in Table 42

L. plantarum, *L. brevis* and *P. pentosaceus* occurred in 100% of bamboo shoot. The prevalence of *L. plantarum* and *L. brevis* was 100%, but that of *P. pentosaceus* was 40-50% of the market and laboratory-made samples.

4.4.3. Optimization of traditional process parameters for mesu production

Table 44 shows the average sensory scores for optimizing fermentation container of young bamboo shoot for mesu production. All the treatments differed significantly ($P < 0.05$) among themselves with respect to every sensory attribute. The treatment B, glass jar with lid as fermenting container, had the highest score.

All the treatments for optimizing temperature of incubation during fermentation of bamboo shoot differed significantly ($P < 0.05$) with respect to taste, flavour and total score (Table 45). However, treatments A and B showed no significant difference ($P < 0.05$) in respect of colour. The treatment B, temperature of incubation at 30°C during bamboo shoot fermentation, had the highest score.

Table 46 shows that all the treatments, considered for optimizing fermentation time, differed significantly ($P < 0.05$) with each other in respect of taste, flavour and total score. However, treatments B and C showed no significant difference ($P < 0.05$) in respect of colour. The treatment B, fermentation time of 10 days, had the highest score.

4.4.4. Successional studies on bamboo shoot during mesu production

Mesu was prepared in the laboratory under optimized conditions for studies on microbial and biochemical changes. The bamboo shoot,

Table 44. Average sensory scores for optimizing fermentation container of bamboo shoot for mesu production

Attributes	Fermentation container		
	A Bamboo vessel	B Glass jar with lid	C Polythene bag
Taste	52.0 ^b (51.8-52.2)	54.8 ^a (54.5-55.0)	44.9 ^c (44.6-45.0)
Flavour	29.5 ^b (29.2-29.8)	32.1 ^a (31.8-32.6)	24.7 ^c (24.3-25.0)
Colour	4.1 ^b (4.0-4.2)	4.6 ^a (4.5-4.7)	2.2 ^c (2.0-2.5)
Total score	85.6 ^b (85.0-86.2)	91.5 ^a (90.8-92.3)	71.8 ^c (70.9-72.5)

Temperature of incubation, 30°C; fermentation time, 10 days

Data represent the means of three replications. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly ($P < 0.05$).

Table 45. Average sensory scores for optimizing temperature of incubation during fermentation of bamboo shoot for mesu production

Attributes	Temperature of incubation (°C)		
	A 20	B 30	C 40
Taste	49.6 ^b (49.0-50.2)	54.4 ^a (54.0-55.0)	45.2 ^c (44.7-45.8)
Flavour	28.2 ^b (28.0-28.4)	32.5 ^a (32.3-32.6)	23.8 ^c (23.6-24.0)
Colour	4.1 ^a (4.0-4.2)	4.6 ^a (4.5-4.7)	2.4 ^b (2.0-2.7)
Total score	81.9 ^b (81.0-82.8)	91.5 ^a (90.8-92.3)	71.4 ^c (70.3-72.5)

Fermentation container, glass jar with lid; fermentation time. 10 days
Data represent the means of three replications. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly (P<0.05).

Table 46. Average sensory scores for optimizing fermentation time during mesu production

Attributes	Fermentation time (days)		
	A 7	B 10	C 15
Taste	46.3 ^c (46.0-46.7)	54.6 ^a (54.3-55.0)	50.1 ^b (49.7-50.5)
Flavour	25.4 ^c (25.0-25.8)	32.2 ^a (32.0-32.3)	29.5 ^b (29.2-29.8)
Colour	3.0 ^b (3.0-3.1)	4.4 ^a (4.0-4.6)	4.0 ^a (4.0-4.0)
Total score	74.7 ^c (74.0-75.6)	91.2 ^a (90.3-91.9)	83.6 ^b (82.9-84.3)

Fermentation container, glass jar with lid; temperature of incubation, 30°C.

Data represent the means of three replications. Ranges are given in parentheses.

Values bearing different superscripts in each row differ significantly ($P < 0.05$).

(*Dendrocalamus hamiltonii* Nees.), after removal of its leaf base, was sliced (1-1.5 cm x 0.5-0.8 cm x 0.5-0.8 cm). A glass jar was tightly packed with the pieces and covered with a lid. They were fermented at 30°C for 10 days. The successional studies were carried out at every 2 days intervals within a range of 0-10 days.

4.4.4.1. Microbial changes

The changes in microflora in bamboo shoot during mesu production are presented in Table 47 and Fig. 56. *Pediococcus pentosaceus* predominated during the early stages of fermentation. Its initial load increased significantly ($P < 0.05$) after 2 days, followed by a steady fall in the count; the decrease was significant ($P < 0.05$) at every 2 days intervals. The load of *Lactobacillus brevis* was maximum on the 4th day, after which there was significant fall ($P < 0.05$) at every 2 days intervals. On the other hand, the population of *L. plantarum* continuously increased from the start of fermentation till the formation of mesu on the 10th day; the increase was significant ($P < 0.05$) at every 2 days intervals till the 6th day.

4.4.4.2. Biochemical changes

Biochemical changes in bamboo shoot during mesu production are shown in Table 48. During fermentation, the mean pH value decreased from 6.35 at 0 day to 3.84 at the end (Fig. 57). On the other hand, the mean titratable acidity increased from 0.04% at 0 day to 0.95% at the end of fermentation. The moisture and total nitrogen contents remained constant throughout fermentation.

Table 47. Changes in microflora in bamboo shoots during mesu production

Fermentation time (days)	cfu(x 10 ⁶)/g fresh weight		
	<i>Pediococcus pentosaceus</i>	<i>Lactobacillus brevis</i>	<i>Lactobacillus plantarum</i>
0	0.2 ^a (0.007-0.4)	0.08 ^{ad} (0.005-0.2)	0.1 ^a (0.006-0.2)
2	18.0 ^b (12.0-28.0)	3.0 ^{bc} (2.0-5.0)	1.2 ^b (0.7-1.5)
4	5.2 ^b (4.0-6.8)	13.0 ^b (8.5-19.0)	8.0 ^c (7.0-10.0)
6	0.2 ^a (0.04-0.4)	1.5 ^c (0.7-3.0)	110.0 ^d (80.0-150.0)
8	0.001 ^c (0.0004-0.002)	0.2 ^d (0.09-0.4)	240.2 ^d (165.0-300.0)
10	0.00001 ^d (0-0.00002)	0.003 ^e (0.00008-0.006)	305.0 ^d (250.0-400.0)

cfu, colony forming units

Data represent the means of five batches of fermentation. Ranges are given in parentheses.

Data were transformed into logarithmic values and then subjected to analysis of variance. Values bearing different superscripts in each column differ significantly (P<0.05).

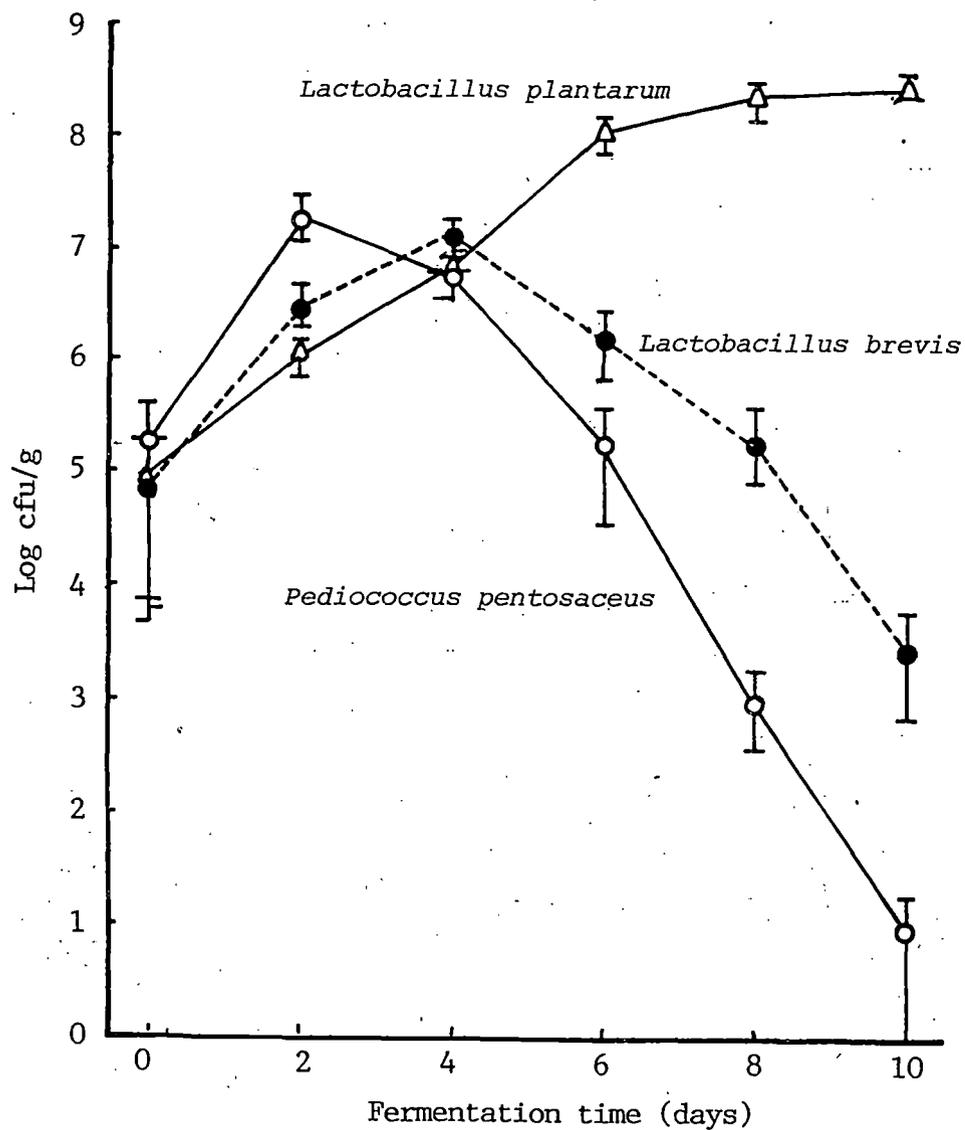


Fig. 56. Changes in microflora in bamboo shoots during mesu production. Values are the means of five batches of fermentation with ranges. Where ranges overlap, bars for the lowest mean are shown to the left; and where three ranges overlap, bars for the highest mean are shown to the right.

Table 48. Biochemical changes in bamboo shoots during mesu production

Fermentation time (days)	pH	Titratable acidity (as % lactic acid)	Moisture (%)	Total nitrogen (% DM)
0	6.35 ^a (6.16-6.50)	0.04 ^a (0.03-0.05)	94.17 ^a (94.12-94.23)	2.78 ^a (2.74-2.82)
2	5.93 ^b (5.80-6.05)	0.22 ^b (0.18-0.27)	94.17 ^a (94.12-94.23)	2.78 ^a (2.74-2.82)
4	5.14 ^c (5.07-5.20)	0.45 ^c (0.40-0.50)	94.17 ^a (94.11-94.23)	2.77 ^a (2.73-2.82)
6	4.38 ^d (4.25-4.50)	0.68 ^d (0.63-0.77)	94.16 ^a (94.11-94.22)	2.77 ^a (2.73-2.82)
8	3.90 ^e (3.75-4.00)	0.92 ^e (0.87-0.97)	94.16 ^a (94.10-94.22)	2.76 ^a (2.73-2.81)
10	3.84 ^e (3.68-3.95)	0.95 ^e (0.90-1.00)	94.16 ^a (94.10-94.22)	2.76 ^a (2.73-2.81)

% DM, percentage on dry matter basis

Data represent the means of five batches of fermentation. Ranges are given in parentheses.

Values bearing different superscripts in each column differ significantly ($P < 0.05$).

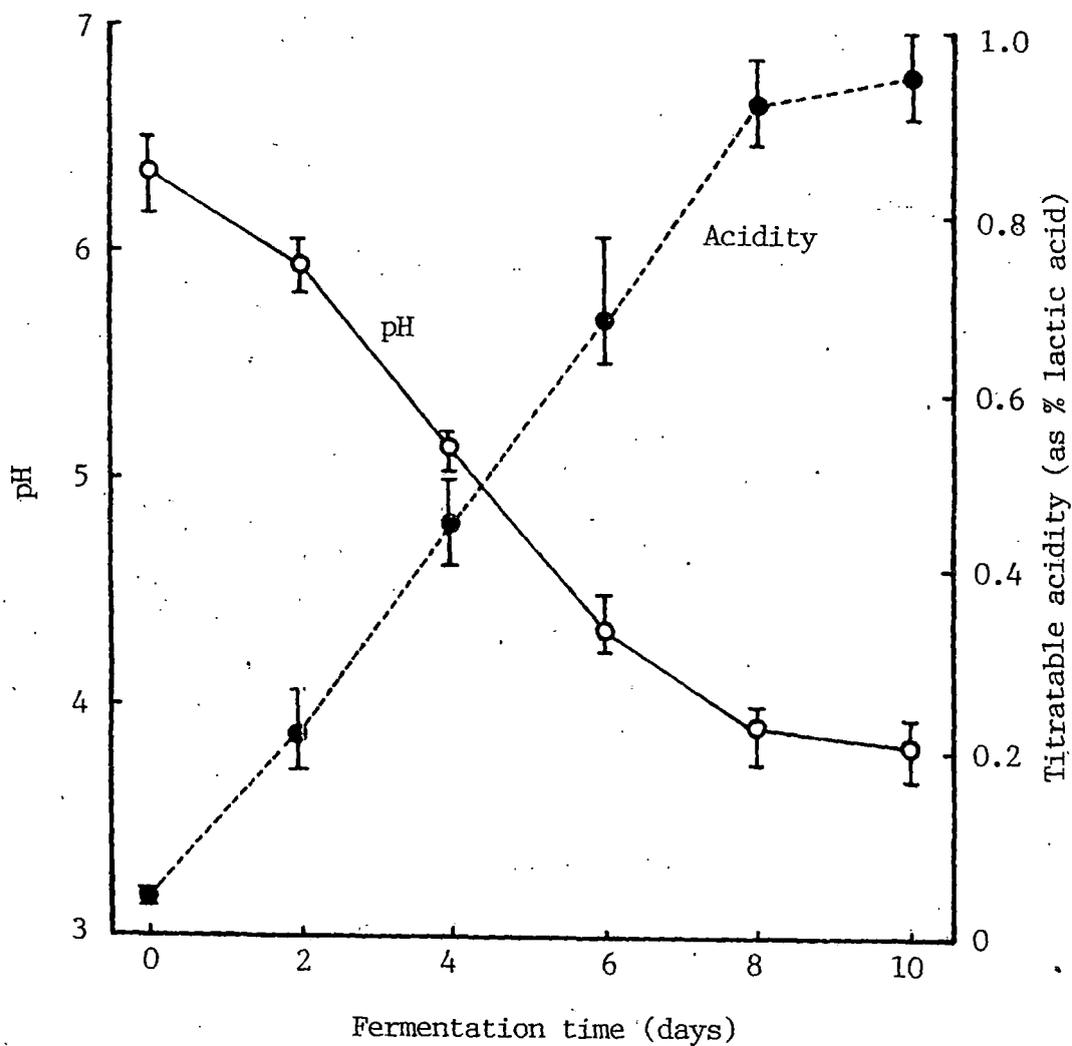


Fig. 57. Changes in pH and acidity in bamboo shoots during mesu production. Values are the means of five batches of fermentation and bars represent changes.

4.5. MURCHA

4.5.1. Proximate composition of market samples

Murcha contains about 13% moisture and 0.7% ash on dry basis. The pH is acidic (Table 49).

4.5.2. Microbial analysis

4.5.2.1. Isolation of microorganisms from market samples

Pure culture colonies developed on MRS agar, GYP-CaCO₃ agar, APT agar, YM agar and PDA, but poorly on nutrient agar. A total of 194 bacterial, 190 yeast and 58 mould strains were isolated from 30 samples of murcha, 10 each from Darjeeling, Kalimpong and Gangtok markets. The bacterial isolates were grouped on the basis of production of acid from D-xylose and lactose (Table 50). The yeast strains were grouped on the basis of colony characteristics and cell morphology (Table 51). All the mould isolates were identical in respect of all the characters studied (Table 52). One representative strain from each group was selected randomly for detailed characterization and identification.

4.5.2.2. Taxonomical studies

4.5.2.2.1. Bacteria

The strains DMu-T1, DMu-T6, KMu-T1 (Fig. 58), KMu-T9, GMu-T1 and GMu-T2 were non-sporeforming, non-motile, Gram positive and catalase negative cocci in tetrads. They were facultative anaerobes and did not produce gas from glucose. These characteristics showed that all those isolates belonged to the genus *Pediococcus*. Their

Table 49. Proximate composition of murcha from different sources

Parameters	Darjeeling market	Kalimpong market	Gangtok market
Moisture (%)	13.0 (11.8-13.6)	12.6 (11.8-13.2)	12.5 (12.0-13.0)
Ash (% DM)	0.70 (0.68-0.73)	0.68 (0.65-0.70)	0.72 (0.68-0.75)
pH	5.20 (4.95-5.70)	5.11 (4.88-5.50)	5.16 (4.82-5.65)

% DM, percentage on dry matter basis

Data represent the means of 10 samples. Ranges are given in parentheses.

Table 50. Selection of representative strains of bacteria isolated from murcha samples^a

Source	Number of strains ^b isolated	Acid produced from		Grouped strains	Representative strains
		D-Xylose	Lactose		
Darjeeling market	64	+	-	38	DMu-T1
		-	+	26	DMu-T6
Kalimpong market	60	+	+	36	KMu-T1
		-	-	24	KMu-T9
Gangtok market	70	-	+	51	GMu-T1
		+	-	19	GMu-T2

^aNumber of samples was 10 from each source.

^bAll isolates were non-motile, non-sporeforming, Gram positive and catalase negative cocci in tetrads.

Table 51. Selection of representative strains of yeasts isolated from murcha samples^a

Source	Number of strains ^b isolated	Colony ^c	Cell shape ^d	Mycelium	Grouped strains	Representative strains
Darjeeling market	60	Ds	O-Cy	True	46	DMu-YD1
		Ss	O-E	Pseudo	14	DMu-YS1
Kalimpong market	58	Ds	O-Cy	True	39	KMu-YD1
		Ss	O-E	Pseudo	19	KMu-YS1
Gangtok market	72	Ds	O-Cy	True	50	GMu-YD1
		Ss	O-E	Pseudo	22	GMu-YS1

^aNumber of samples was 10 from each source.

^bAll the isolates reproduced by multilateral budding.

^cDs, dusty surface; Ss, smooth surface

^dO-Cy, oval to cylindrical; O-E, oval to ellipsoidal

Table 52. selection of representative strains of moulds isolated from murcha samples^a

Source	Number of strains ^b isolated	Grouped strains	Representative strains
Darjeeling market	15	15	DM ₁ -M ₁
Kalimpong market	21	21	KM ₁ -M ₁
Gangtok market	22	22	GM ₁ -M ₁

^aNumber of samples was 10 from each source.

^bAll the isolates had mycelia without septation, rhizoid and stolon, circinate borne sporangia containing oval spores.

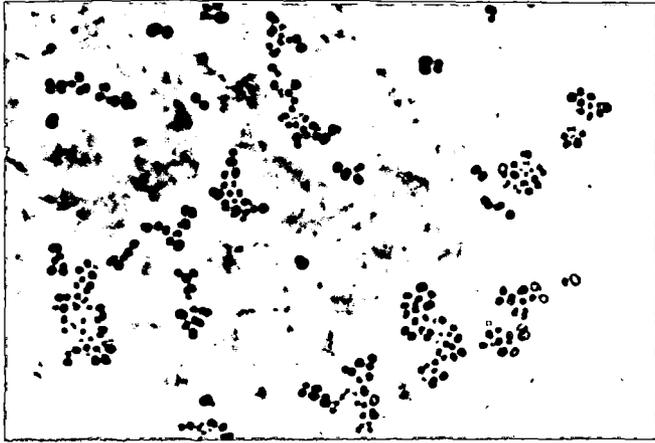


Fig. 58. *Pediococcus pentosaceus* KMu-TI (APT agar, 3 days, 30°C) isolated from murcha; showing coccal cells in tetrads; phase contrast micrograph (x 1120)



Fig. 59. *Saccharomycopsis fibuligera* KMu-YD1 (Slide culture on PDA, 2 days, 28°C) isolated from murcha, showing oval to cylindrical cells with true mycelia; phase contrast micrograph (x 800)

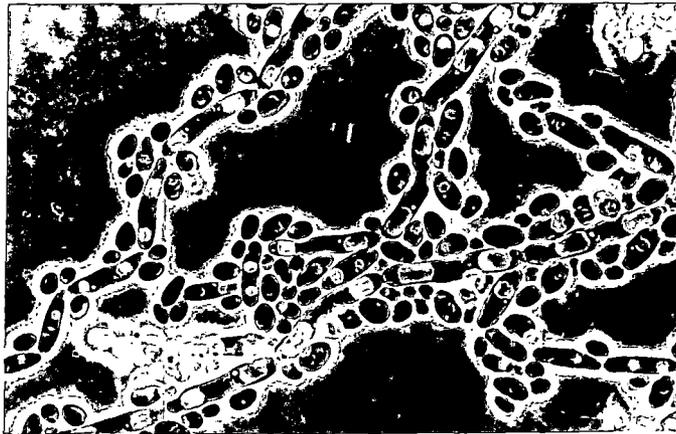


Fig. 60. *Pichia anomala* KMu-YS1 (Slide culture on PDA, 2 days, 28°C) isolated from murcha, showing oval to ellipsoidal cells with pseudomycelia; phase contrast micrograph (x 800)

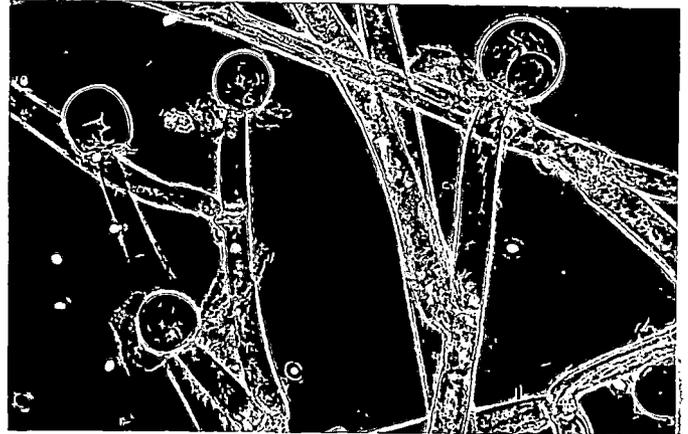


Fig. 61. *Mucor circinelloides* KMu-M1 (PDA, 2 days, 28°C) isolated from murcha showing stalked sporangia with spores; phase contrast micrograph (x 800)

detailed morphological and physiological characteristics are shown in Table 53. Following the taxonomic key of Garvie (1986b), the representative strains were identified as *Pediococcus pentosaceus* Mees.

4.5.2.2.2. Yeasts

The morphological and physiological characteristics of the representative strains isolated from market samples of murcha are shown in Table 54. Colonies of the three strains including DMu-YD1, KMu-YD1 and GMu-YD1 had dusty, dry surface and horn-like projections made up of many strands of mycelia when grown on agar plates. The cells were oval to cylindrical and showed true, septate hyphae with blastoconidia. There were 2-4 hat-shaped ascospores in each ascus. None of these strains could reduce nitrate and utilize cellobiose. But, all of them fermented starch. On the other hand, the colonies of the other three strains including DMu-YS1, KMu-YS1 and GMu-YS1 had glistening surface with entire margin on agar plates. The cells were oval to ellipsoidal. There were 1-4 hat-shaped ascospores in each ascus. Those strains reduced nitrate to nitrite.

According to the criteria laid down by Barnett et al. (1983) and Kreger-van Rij (1984) the strains DMu-YD1, KMu-YD1 (Fig. 59) and GMu-YD1 were identified as *Endomyces fibuliger* Linder, and the strains DMu-YS1 (Fig. 60), KMu-YS1 and GMu-YS1 were identified as *Hansenula anomala* (Hansen) Sydow and Sydow. *Endomyces fibuliger* Lindner (= *Endomyces lindneri* Saito) and *Hansenula anomala* (Hansen) Sydow and Sydow are now considered as *Saccharomycopsis fibuligera*

Table 53. Characteristics of representative strains of tetrad bacteria isolated from murcha samples

Parameters	DMu-T1	DMu-T6	KMu-T1	KMu-T9	GMu-T1	GMu-T2
Cell morphology						
Shape	Coccus	Coccus	Coccus	Coccus	Coccus	Coccus
Arrangement	Tetrad	Tetrad	Tetrad	Tetrad	Tetrad	Tetrad
Size: diameter (μm)	$\emptyset.7-\emptyset.7$	$\emptyset.6-\emptyset.7$	$\emptyset.6-\emptyset.7$	$\emptyset.6-\emptyset.7$	$\emptyset.6-\emptyset.7$	$\emptyset.6-\emptyset.7$
Spore	-	-	-	-	-	-
Motility	-	-	-	-	-	-
Gram reaction	+	+	+	+	+	+
Catalase	-	-	-	-	-	-
O ₂ requirement	F ^a	F	F	F	F	F
Gas from glucose	-	-	-	-	-	-
Hydrolysis of						
Fat	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-
Arginine	+	+	+	+	+	+
Esculin	+	+	+	+	+	+
Growth in						
4 and 6.5%	+	+	+	+	+	+
18.0%	-	-	-	-	-	-
Growth in pH 4.2-8.5	+	+	+	+	+	+
Growth at						
35 and 40°C	+	+	+	+	+	+
50°C	-	-	-	-	-	-
Optimum temperature						
for growth (°C)	30	30	30	30	30	30

Parameters	DMu-T1	DMu-T6	KMu-T1	KMu-T9	GMu-T1	GMu-T2
Reduction of						
nitrate to nitrite	-	-	-	-	-	-
Reduction of nitrite to N ₂	-	-	-	-	-	-
Indole production	-	-	-	-	-	-
Acid produced from						
L-Arabinose	+	+	+	+	+	+
Cellobiose	+	+	+	+	+	+
Fructose	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+
D-Galactose	+	+	+	+	+	+
Lactose	-	+	+	-	+	-
Maltose	+	+	+	+	+	+
Mannitol	-	-	-	-	-	-
Mannose	+	+	+	+	+	+
Melibiose	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-
D-Ribose	+	+	+	+	+	+
Sucrose	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-
Xylose	+	-	+	-	-	-
Trehalose	+	+	+	+	+	+
Starch	-	-	-	-	-	-

^aFacultative anaerobe

Table 54. Characteristics of representative strains of yeasts isolated from murcha samples

Parameters	DMu-YD1	KMu-YD1	GMu-YD1	DMu-YS1	KMu-YS1	GMu-YS1
Cell morphology						
Shape	O-Cy ^a	O-Cy	O-Cy	O-Cy	O-Cy	O-Cy
Size						
Width (µm)	3.5-5.2	3.5-5.7	3.5-5.5	2.4-3.5	2.2-3.5	2.2-3.5
Length (µm)	5.2-8.7	5.0-8.5	5.0-8.7	3.5-5.2	3.5-5.5	3.5-5.3
Mycelium	True	True	True	Pseudo	Pseudo	Pseudo
Vegetative reproduction						
Budding, multilateral	+	+	+	+	+	+
Blastoconidia	+	+	+	-	-	-
Sexual reproduction						
Ascus	Evanescent	Evanescent	Evanescent	Evanescent	Evanescent	Evanescent
Ascospore	2-4, hs ^b	2-4, hs	2-4, hs	1-4, hs	1-4, hs	1-4, hs
Reduction of nitrate to nitrite	-	-	-	w ⁺ ^c	w+	w+
Reduction of nitrite to N ₂	-	-	-	-	-	-
Growth at 37°C	+	+	+	+	+	+

Parameters	DMu-YD1	KMu-YD1	GMu-YD1	DMu-YS1	KMu-YS1	GMu-YS1
Fermentation						
D-Glucose	+	+	+	+	+	+
D-Galactose	-	-	-	-	+	-
Sucrose	+	+	+	+	+	+
Maltose	-	+	+	+	+	+
Cellobiose	-	-	-	+	+	+
Raffinose	+	-	+	+	+	-
Starch	+	+	+	-	-	-

^aOval to cylindrical

^bHat-shaped

^cWeak positive

(Lindner) Klöcker and *Pichia anomala* (Hansen) Kurtzman, respectively (Hesseltine and Kurtzman 1990).

4.5.2.2.3. Moulds

The mycelia of the strains DMu-M1, KMu-M1 (Fig. 61) and GMu-M1 were aseptate without any rhizoid and stolon. The sporangiophores were repeatedly branched with many sporangia borne circinately. The sporangial wall mostly deliquesced. Sporangiospores were oval. All grew at 37°C. Following the taxonomic keys of Zycha and Siepmann and Hesseltine and Ellis (1973), the mould strains were identified as *Mucor circinelloides* van Tieghem.

4.5.2.3. Microbial load of market samples

The average microbial load studied in 10 samples of murcha collected from each of Darjeeling, Kalimpong and Gangtok markets are presented in Table 55 and Fig. 62. The load of *Pediococcus pentosaceus* occurred at a level of $2-42 \times 10^7$ cfu/g in market murcha. Among the yeasts, *Saccharomycopsis fibuligera* showed higher load in murcha which ranged between $4-68 \times 10^7$ cfu/g; *Pichia anomala* occurred at a level of $0.2-7.2 \times 10^7$ cfu/g. *Mucor circinelloides*, present in all the murcha samples, were at a level of $0.1-4.1 \times 10^7$ cfu/g.

Table 56 shows that all the four types of microorganisms were present in 100% of the market samples.

Table 55. Microbial load of murcha from different sources

Microorganisms	cfu ($\times 10^7$)/g fresh weight		
	Darjeeling market	Kalimpong market	Gangtok market
Bacteria			
<i>Pediococcus pentosaceus</i>	18.8 (2.0-38.5)	20.0 (2.0-41.0)	21.0 (5.0-42.0)
Yeasts			
<i>Saccharomycopsis fibuligera</i>	35.8 (4.0-57.0)	39.0 (4.5-68.0)	37.0 (6.2-64.0)
<i>Pichia anomala</i>	3.0 (0.2-7.0)	3.0 (0.2-7.2)	2.8 (0.2-5.7)
Moulds			
<i>Mucor circinelloides</i>	2.1 (0.1-3.2)	2.0 (0.1-3.0)	2.3 (0.2-4.1)

cfu, Colony forming units

Data represent the means of 10 samples. Ranges are given in parentheses.

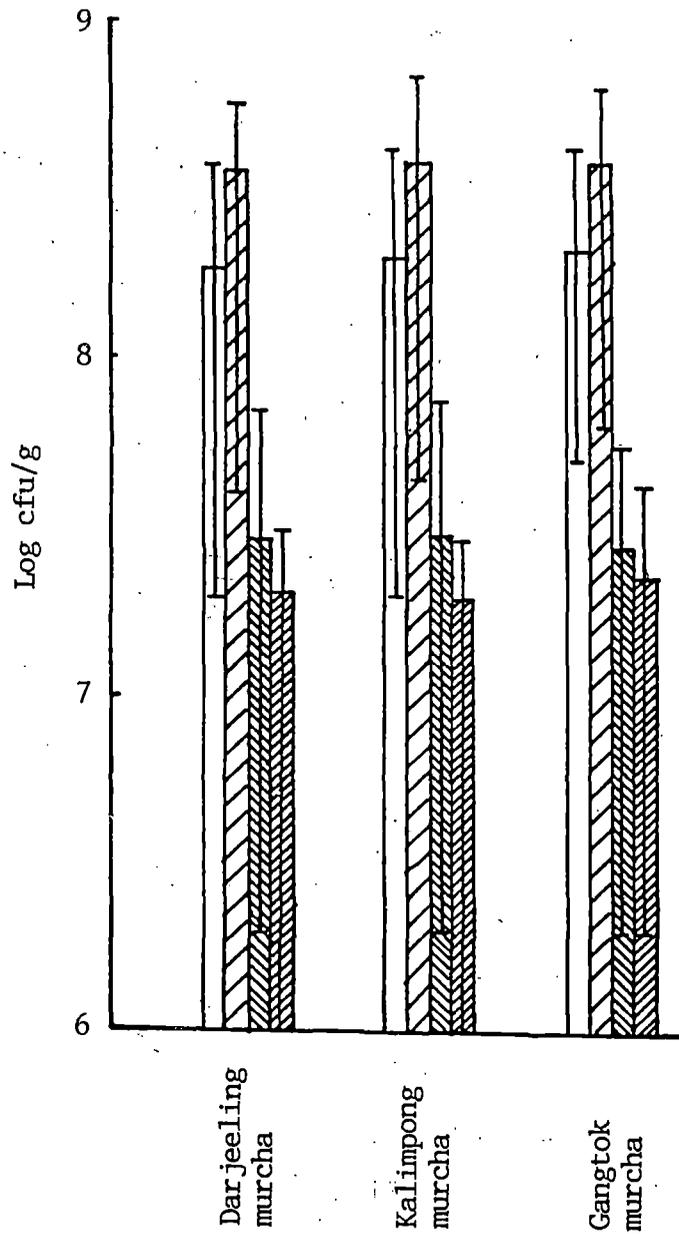


Fig. 62. Microbial load of murcha from different sources. Values are the means with ranges of 10 samples. *Pediococcus pentosaceus*,  ; *Saccharomycopsis fibuligera*,  ; *Pichia anomala*,  ; *Mucor circinelloides*, 

Table 56. Prevalence of various microorganisms in murcha from different sources

Microorganisms	Positive samples (%) ^a		
	Darjeeling market	Kalimpong market	Gangtok market
Bacteria			
<i>Pediococcus pentosaceus</i>	100	100	100
Yeasts			
<i>Saccharomycopsis fibuligera</i>	100	100	100
<i>Pichia anomala</i>	100	100	100
Moulds			
<i>Mucor circinelloides</i>	100	100	100

^aExpressed on the basis of a total of 10 samples studied as in Table 55

5. DISCUSSION

The fermented foods, traditionally used in these regions, have certain ethnic values and social importances. Kinema is not traditionally consumed by the Brahmins, regarded as the most elite caste of the Hindus. Although the reason is not documented, it is believed that the Brahmins usually regard kinema as 'basi', meaning stale.

The sun-drying of gundruk and sinki is a remarkable step in the traditional method. They can be stored easily for several months and consumed during monsoon when fresh vegetables are scarce. Dried gundruk and sinki are comparatively less in weight than the weight of fresh substrates, and can therefore be carried easily while travelling. Previously, there was no transportation in these regions, and people had to walk long distances, even weeks. They used to carry gundruk and sinki during their journey to feed themselves. Carrying gundruk and sinki, and sometimes sukako masu, is still a common practice among the people while travelling for long distances. Because of the acidic taste, gundruk and sinki are said to be good appetizers, and people use them for remedies from indigestion.

Consumption of meat is very expensive for the poor rural people. They slaughter domestic animals usually on special occasions, like festivals and marriages. During the Dashera, one of the greatest festivals of these regions, usually at every house, goats are ritually sacrificed to please the Goddess Durga. After the Puja, the fresh meat is cooked and eaten. The remainder is preserved by smoking to make sukako masu for future use. The Bhutias and the Lepchas inhabiting the high altitudes of Sikkim

also slaughter yaks occasionally, consumed the fresh meat and the rest is preserved by smoking.

Celebration of festivals with shel roti is a custom of the Nepalis. Preparation of sweets, like in other Indian communities, is not a practice in these regions. Instead, shel roti, a slightly sweet item, serves as confectionary during festivals. Besides its consumption as a food, dahi is used by the Nepalis as an adhesive for rice grains and colour to make 'tika' which is applied on the foreheads of the younger members of the family by their elders during festivals and marriages.

Murcha makers are restricted to the Limbu and Rai castes of the Nepalis, and the Lepchas. Traditionally, the Limbu and Rai are known as 'matwalis', meaning alcohol drinkers. The preparation of murcha is done by women of these castes. In order to keep this art secret, murcha is prepared usually at night. The trade in murcha is protected as a hereditary right of these castes. This may be the reason of adopting the murcha preparation only by certain ethnic groups. Murcha makers believe that during its preparation, addition of spices, such as chillies and ginger in rice flour is necessary to drive away evils that spoil the product. The rationale behind this is to check the growth of undesirable microorganisms that may inhibit the growth of fermenters. The studies of Soedarsono (1972), Koedam (1977) and Frazier and Westhoff (1978) reveal that certain spices inhibit many undesirable microorganisms at the time of fermentations.

Jnard is the most common drink in these regions. Guests are welcome usually with jnard in bamboo vessels (toongbas). The custom of serving jnard to guests in Sikkim has been well-described

by Hooker (1854). Alcoholic beverages are commonly used for worshipping Gods and Goddesses, a custom practised by some ethnic groups of the Nepalis, the Bhutias and the Lepchas. Solemnizing marriages with alcoholic beverages is a common practice among different ethnic groups of these regions. During marriages, priests offer small amounts of raksi to newly wedded couples to signify that they are matwalis.

Mesu is not so popular in Darjeeling subdivision. It may be due to non-availability of bamboo shoots because of high altitudes and vast tea plantations. It is not even sold in local markets of Darjeeling. Similarly, in most areas of North district, and a few places in the West and South districts of Sikkim, bamboos are not grown. Hence, the preparation of mesu is restricted to a few places. Limitation of khalpi consumption to a few places may be due to the short shelf-life of the pickle. Growing cucumber is also restricted to a few places in lower altitudes. Masayura is also seen to be prepared only in a few places where black grams are cultivated in the lower altitudes of these regions.

5.1. KINEMA

The preparation of kinema is very similar to that of natto. In itohiki-natto whole soya beans are used for fermentation, and in hikiwari-natto dehulled soya beans cracked into 2 to 4 pieces are used (Ohta 1986). In kinema, always the whole soya beans are used. Natto prepared from whole soya beans is reported to be superior in palatibility and higher in ammonia content than natto made from dehulled soya beans (Sakurai and Nakano 1961). Like in most

Oriental fermentations of soya beans, soaking in water and cooking, and then discarding the water are the important steps in kinema preparation. Soaking and cooking of soya beans help to inactivate and leach out some undesirable factors such as phytic acid (Toma and Tabekhia 1979; Chang et al. 1977), flatus-causing oligosaccharides (Wang et al. 1979) and trypsin inhibitors (Albrecht et al. 1966), and the water of which is then discarded (Hesseltine 1985b). The heating process also reduces the *in situ* microbial contaminants, especially non-sporulating bacteria and moulds (Wang and Hesseltine 1981).

Some of the steps in kinema preparation do not resemble to those in natto, and thus make kinema a unique non-salted soya bean fermented product. The cooked beans are lightly crushed to dehull most of the seeds. But, fermentation is carried out with the kernels as well as the seed coats. While natto is consumed as is with shoyu (Kiuchi et al. 1976; Fukushima 1979), kinema is always fried in oil and made to curry. The practice of frying kinema may have developed to drive out the unpleasant ammoniacal flavour which masks the pleasant and persistent nutty flavour.

Because of soaking and cooking of soya beans prior to fermentation, kinema had a high (ca 62%) moisture content. A marked decrease in the fat content of kinema compared to raw soya beans was due to lipolytic activities of the microorganisms during kinema production with concomitant increase in free fatty acidity. All the four types of isolates had the ability to hydrolyse fat. The slight increase of carbohydrate content in kinema was in agreement with the report in natto and tempe (Ohta 1986; Wang

1986a,b). The higher ash content of market samples compared to the laboratory-made kinema is likely due to addition of firewood ash during kinema production. Ash was not added during the preparation of kinema in the laboratory. Addition of ash during kinema preparation is a practice in a few places. This may be for reducing any bad odour, as practised in daddawa (Campbell-Platt 1980). Although the acidity in kinema increased by about 10 fold over raw soya beans, the product had a high pH value (7.9). This was due to the high buffering capacity of the legume beans and the proteolytic activities of *Bacillus subtilis* leading to ammonia release, characteristic of most vegetable protein fermentations (Hesseltine 1965). The energy value of 1.9 MJ found per 100 g dry matter of kinema was very near to the energy values in natto (2.0 MJ), tempe (1.8-1.9 MJ) and daddawa (2.1-2.3 MJ) (Campbell-Platt 1987).

Two types of bacteria and two types of yeasts were isolated from market samples of kinema. Among the bacteria, one type included sporeforming rods and the other, non-sporeforming cocci. Following the taxonomic keys of Norris *et al.* (1981), the sporeforming rods were assignable to *Bacillus subtilis*, since they could produce catalase, amylase and acetoin, but were unable to grow anaerobically and to change the pH of VP broth to <6.0. However, they did not share the character of nitrate reduction of *B. subtilis*. Next to *B. subtilis*, the isolates had a close resemblance with *B. pumilus* and *B. coagulans*. The isolates differed from *B. pumilus* with respect to hydrolysis of starch and change pH of VP broth to <6.0, and from *B. coagulans* with respect to growth in anaerobic agar, 7% NaCl and change the pH of VP broth to <6.0.

Following the detailed morphological and API test profiles of *Bacillus* (Logan and Berkeley 1984), the sporeformers showed similarities mostly with *B. subtilis* and *B. coagulans*. They differed from *B. subtilis* with respect to production of tryptophan desaminase, and acid from arbutin, salicin and cellobiose. Presence of tryptophan desaminase and central or paracentral position of spores in the kinema isolates made them different from *B. coagulans*. They differed from *B. pumilus* with respect to a number of characters including position of spores, β -galactosidase and tryptophan desaminase content, and production of acid from D-galactose, N-acetylglucosamine, arbutin, salicin, cellobiose, inulin, starch, glycogen and β -gentibiose.

According to the criteria laid down by Claus and Berkeley (1986), those isolates were not unequivocally assignable to any of the species of *Bacillus*. They differed from *B. subtilis* with respect to position of spore, utilization of citrate, reduction of nitrate and growth at 55°C. Inability to grow in anaerobic agar, at 10°C and to change the pH of VP broth to <6.0, but ability to hydrolyse gelatin and to grow in 5 and 7% NaCl, and non-requirement of growth factors made the isolates differentiated from *B. coagulans*. Another closely related species, *B. pumilus* differed from them with regard to position of spore, utilization of citrate, hydrolysis of starch, and growth at 10, 50 and 55°C.

Although the sporeforming isolates have tentatively been designated as members of *B. subtilis*, they need the status of a new species.

The spherical-celled strains of *Streptococcus* differed from its allied genus *Leuconostoc* by the absence of gas production from glucose in the former (Garvie 1986a). These strains belonged to the group enterococci due to their growth at 6.5% NaCl and pH 9.6, the characters commonly used to segregate the enterococci from other streptococci (Mundt 1986). Of the four species in this group, two are obligate pathogens of animals (Hesseltine and Ray 1988), and thus two species, *S. faecalis* and *S. faecium*, remain. Schleifer and Klipper-Bälz (1984) transferred these two species from *Streptococcus* to a new genus *Enterococcus*. The production of acid from L-arabinose, arbutin and melibiose, non-production of acid from melezitose and α -haemolysis on blood agar by these strains did not suggest their inclusion in *E. faecalis*. The detailed characteristics revealed that all these kinema strains belonged to *Enterococcus faecium* (Orla-Jensen) Schleifer and Klipper-Bälz.

Among the two types of yeasts isolated from market samples, one type was absent in the kinema prepared in laboratory. The type which was present in both market and laboratory-made samples had cream-coloured, ellipsoidal cells with multilateral budding, pseudomycelia and no sexual reproduction. On the basis of growth, oxidation and fermentation of a host of carbohydrates, in addition to the above mentioned cultural and morphological characteristics, the strains of this type were identified as *Candida parapsilosis* (Ashford) Langeron and Talice. The other type had true (septate) hyphae with arthroconidia, splitting cells and no sexual reproduction. The strains of this type were assigned to *Geotrichum candidum* Link.

The 100% prevalence of *Bacillus subtilis* and *Enterococcus faecium* in a total of 50 samples of kinema indicate their possible involvement in the production of kinema. Microbial analysis of raw soya beans exhibited the presence of *B. subtilis* only. This finding was in agreement with the statement of Hesselstine (1983b) that *B. subtilis* is commonly found on soya beans. The involvement of *B. subtilis* has been reported in several soya bean-based foods, such as natto (Sakurai 1960; Hesselstine 1965; Ohta 1986) and thua-nao (Sundhagul *et al.* 1972), and African locust bean (*Parkia biglobosa* Benth.) food, daddawa (Odunfa 1981). The presence of *E. faecium* has been reported in gari, a popular cassava (*Mannihot esculenta* Crantz.) tuber fermented food of West Africa (Abe and Lindsay 1978). This species is present in several non-starch foods but the source is not from faecal contamination (Schleifer and Klipper-Bälz 1984). The lower frequency of *Candida parapsilosis* and *Geotrichum candidum* in kinema indicates that they may be opportunistic organisms, having no involvement in fermentation. The aerial contamination by yeasts in foods has been reported by Sandhu and Waraich (1981). *Candida parapsilosis* has been found in ragi (Saono *et al.* 1974), and *Geotrichum candidum* has been reported in many fermented foods, such as gari, cassava bread and kenkey of Africa, filmjolk of Europe, poi of Pacific Islands, pozol of Mexico and torani of Indian Subcontinent (Campbell-Platt and Cook 1989).

The traditional methods of kinema preparation varied in details from home to home, and as a result, the quality of the product was very inconsistent. In order to produce a product of reproducible quality with flavour and texture acceptable and

attractive to larger groups, and to scale up, the process parameters were optimized by sensory evaluation. Thinly perforated polythene bag was determined as the optimum wrapping material of fermenting soya bean seeds. The fermentation of beans either on a polythene sheet or within a tightly packed polythene bag gave significantly unsatisfactory ($P < 0.05$) result. Although *Bacillus subtilis* did not grow anaerobically, the counts obtained under aerobic condition and reduced oxygen condition in candle jar were the same. The colonies, however, spread better under reduced oxygen condition producing larger sized colonies. The environment provided in the traditional process as already described and in the perforated polythene bag as optimized would, in addition to providing a semi-anaerobic environment, also keep the beans moist and thus help the spreading of motile *B. subtilis* (Gordon et al. 1973) and the growth of facultative anaerobic *E. faecium*. It was further noted that soaked, but uncooked, soya beans produced a poor product, not only in respect of body and texture, but also regarding flavour and colour. The cooking process actually reduces the *in situ* undesirable microorganisms (Wang and Hesselstine 1981). On the other hand, 25 min cooking in 0.7 kg/cm^2 steam pressure seems to be high to kill even the important sporeformer which resulted in significant deterioration ($P < 0.05$) of the product. The optimum time was 10 min and possibly this was enough to reduce the load of undesirable microorganisms developed during soaking, without disturbing at all the load of heat resistant sporeformers. The load of *B. subtilis* in raw soya beans as well as in the beans immediately after cooking was $10^6/\text{g}$ fresh weight. Moreover, this

temperature-time treatment provided the optimum softness of the seeds for fermentation. The temperature of incubation at 28°C resulted in a very slow fermentation rate and might provide the optimum milieu for growth of yeasts, making the product rancid. At 48 h fermentation time, 37°C was the optimum temperature for fermentation. The fermentation rate at 45°C was very high and after 48 h of fermentation, the product was over-fermented and resulted in significant deterioration ($P < 0.05$). Due to maintenance of high temperature for 48 h, the desirable viscous substance formed become dried. Traditionally, the fermentation time varies from 1-3 days. In this study, one day was not enough to complete fermentation at 37°C; may be the growth of the fermenting microorganisms did not increase considerably to cause desirable biochemical changes. After 3 days' fermentation at 37°C, the product became dried-up and had a very strong ammoniacal and rancid odour resulting in significantly low ($P < 0.05$) sensory score. Hence, the optimum fermentation time at 37°C was 48 h.

A study on microbial and biochemical changes accompanying soya bean fermentation producing kinema under optimized conditions was then carried out.

The initial level ($ca 10^6/g$) of *Bacillus subtilis* even at the onset of fermentation was due to their presence on raw soya beans and their passage through soaking and cooking treatments, the processes which did not reduce their load. Although *Enterococcus faecium* and *Candida parapsilosis* were not found on raw saw beans, their detection even at the start of fermentation indicates their entry through tap water. In many foods, *Enterococcus faecium* appears as non-faecal contaminant (Mundt 1986). This species occurs

predominantly in soak water of soya beans during tempe preparation and is responsible for acidification of soak water (Mulyowidarso et al. 1989). *Candida parapsilosis* is present in water (Barnett et al. 1983). Unlike 20-30 min cooking time in steam pressure for soya beans in natto preparation (Ohta 1986), the cooking time for beans in kinema preparation is 10 min only. In the latter process, probably the reason is not to destroy the *in situ* sporeformer, the main or possibly the sole fermenting organism, but to reduce the number of others, because kinema is naturally fermented.

From the onset of fermentation, the logarithm of the number of *B. subtilis* cells increased significantly ($P < 0.05$) at every 8 h intervals till the end of fermentation at 48 h. Although, initially the load of *E. faecium* was 40 times less than the load of *B. subtilis*, at the end of fermentation the load of *Enterococcus* was only 5 times less than that of *Bacillus*. This indicates that the growth rate of *Enterococcus* was even higher than that of *Bacillus*, even in alkaline pH. This is not surprising, because *E. faecium* is able to grow even at pH 9.6. Although present at a much lower load, *C. parapsilosis* increased significantly ($P < 0.05$) at every 8 h intervals till 32 h. During the first 16 h, as long as the organisms were growing exponentially, the pH of the fermenting beans went down from 6.94 to 6.64. During the first 8 h, there was no significant rise ($P < 0.05$) in free fatty acid and non-protein nitrogen contents. Therefore, it seems likely that sugars, not proteins or fats, were initially used as substrates for metabolism and growth. Cooked soya beans contain sucrose, raffinose and stachyose (Steinkraus 1983a), and *B. subtilis* was capable of producing acid from sucrose, raffinose and their hydrolysing

products, glucose and fructose. *Enterococcus faecium* was capable of producing acid from galactose, another hydrolysing product of raffinose. *Candida parapsilosis* was capable of utilizing sucrose and all the hydrolytic products of raffinose. However, after 16 h the pH started rising up significantly ($P < 0.05$) at every 8 h intervals till 40 h. This was probably due to proteolytic activities of the microorganisms. While *B. subtilis* was capable of hydrolysing protein (casein and gelatin), *E. faecium* was unable to hydrolyse them. Presumably, the protease produced by *B. subtilis* degraded soya proteins which resulted in significant increase ($P < 0.05$) of non-protein nitrogen content at every 8 h intervals, starting from 8 h till the end of fermentation. In daddawa, which is similar to kinema, the pH of fermenting beans drops from 6.7-6.8 to 5.2-5.4 before rising again to final 7.0-8.0. Hydrolysis of protein to produce amines and ammonia through peptides and ammonia is responsible for this final change in pH (Odunfa 1985b; Campbell-Platt 1987). The production of ammonia is also common in natto (Hesseltine and Wang 1967; Ohta 1986) and tempe (Hesseltine 1965). Due to lipolytic activities of the microorganisms, the fat content in soya beans is significantly degraded ($P < 0.05$) to free fatty acids at every 8 h intervals since 8 h till the end of fermentation. Such increase in free fatty acidity is reported in tempe (Wagenknecht et al. 1961) and natto (Ohta 1986).

Organoleptically, the monoculture fermentation of soya beans by *B. subtilis* produced the best kinema because of a pleasant nutty flavour and highly sticky texture of the product. The unique flavour and mucilaginous texture are observed in natto, fermented

by *B. subtilis* only (Hayashi *et al.* 1971; Ohta 1986). The flavour of natto originated from the hydrolysis of soya bean proteins to peptides and amino acids, fermented by pure culture of *B. subtilis* (Ohta 1986). Sterile soya beans inoculated with *E. faecium* only caused almost no fermentation, giving the least score in respect of every sensory attribute. Since *E. faecium* was unable to hydrolyse protein, soya beans were not fermented to kinema by this organism alone. Again, the product produced by any combination of yeast with *B. subtilis* had significantly less ($P < 0.05$) scores than kinema produced by *B. subtilis* alone or its combination with *E. faecium*. This was due to rancid flavour developed during the fermentation. The sensory analysis showed that yeasts had no role in fermentation of soya beans to make kinema. The combination of *B. subtilis* and *E. faecium* gave the satisfactory score, although it differed significantly ($P < 0.05$) with the product prepared solely by *B. subtilis*. The result of this experiment led us to conclude that *B. subtilis* is the sole fermenting organism for the production of kinema.

Bacillus subtilis DK-W1 was then selected for the improvement of kinema production by monoculture fermentation. Since the optimum temperature for growth of *B. subtilis* was 45°C, an attempt was made to determine the optimum time for fermentation at that temperature following *Bacillus* inoculation. The 18 h period was determined as the optimum period for fermentation at 45°C. Organoleptically, that kinema had a very pleasant nutty flavour associated with a mild ammoniacal odour and a highly sticky texture, scoring to the excellent level. The shorter time obtained for optimum fermentation ^{eliminates} the chance of growth of contaminants and

accumulation of high levels of ammonia which adversely affects the nutritional quality and palatability (Sakurai 1960; Odunfa and Adewuyi 1985).

In monoculture preparation of kinema, the load of *B. subtilis* increased significantly ($P < 0.05$) after 6 h and at every 3 h intervals thereafter till 15 h. Between 6-9 h of fermentation, the surface of the beans was covered with a whitish mass due to growth of *B. subtilis* and subsequent sporulation. This observation in kinema is similar to that in natto. In natto preparation, within 6-8 h of incubation at 40-43°C, the surface of the beans changes to whitish colour due to growth of *B. subtilis* and there is a fermentation odour (Ohta 1986). During kinema production, the beany flavour gradually diminished and finally came to halt after 6 h, giving a flat flavour. After 9 h, a pleasant nutty flavour appeared, and after 15 h the odour of ammonia started to develop.

The monoculture fermentation of *B. subtilis* for kinema production had many advantages over the conventional natural fermentation. Firstly, the load of sporeformer in monoculture could be manipulated to make a good start. Although load of *B. subtilis* in monoculture fermentation was made almost equal to that in natural fermentation, its growth was greatly favoured in the monoculture fermentation where the counts greatly exceeded the counts in natural fermentation in the corresponding intervals. Secondly, the desirable fermentation was completed within a much shorter period of incubation with the organoleptically excellent product development. Thirdly, at high temperature and short period of incubation, the chance of the growth of contaminants was remote.

Fourthly, because of the controlled fermentation, the biochemical changes up to 18 h were desirable. The pH initially fell down significantly ($P < 0.05$) within 6 h of incubation and then increased significantly ($P < 0.05$) up to 7.50 at the end of fermentation. This indicates that the production of ammonia during monoculture fermentation was not so high as in natural fermentation. The free fatty acid content in kinema prepared by monoculture fermentation was much less than that in kinema prepared by the conventional method. The monoculture fermentation made the kinema with no undesirable odour. The desirable increase in soluble nitrogen and non-protein nitrogen within 18 h of fermentation time, comparable to that of conventional method, gave the product acceptable and probably contributing the improved digestibility.

The kinema production is a solid state fermentation process. The high moisture content, however, indicates its very short shelf-life. To improve its keeping quality the local people often sun dry kinema. Although no toxicological study has been conducted with kinema, information regarding the toxic effect due to consumption of kinema was not available. The fact that moulds are not found in this fermentation makes kinema safe from the risk of mycotoxins. *Bacillus subtilis* is non-pathogenic and therefore a safe organism on vegetable proteins (Odunfa 1981).

The ammoniacal odour with typical kinema flavour is always acceptable to kinema consumers. However, some market samples analysed had undesirable rancid odour. This may be due to liberation of free fatty acids in higher amount by lipolytic activities of microflora present in kinema. Young and Wood (1977)

observed that the liberation of free fatty acid in higher amount is undesirable because of its own taste and developing rancidity in the product. Kiuchi *et al.* (1976) observed that high amount of free fatty acid in hama-natto gives the product a strong harsh taste. However, Wang *et al.* (1975) identified the free fatty acids liberated during fermentation of soya beans by *Rhizopus oligosporus* as antitryptic factors.

Although *E. faecium* did not add any sensory quality to the *Bacillus* fermentation of soya beans, it was always encountered in naturally fermented kinema. Its role, if any, needs to be investigated. Presence and growth of yeast during kinema preparation are associated with the development of rancidity in the products. In fact, *B. subtilis* is the sole fermenting organism in kinema preparation.

5.2. SINKI

Unlike many Oriental fermented vegetables including takana, nozawana, hiroshimana and kimchi, sinki is a non-salted fermented vegetable product. Sinki has a soury taste and acidic with a typical flavour. It is a naturally fermented product of radish tap roots.

The fresh sinki had mean moisture content of 93.5%, slightly less than the value in the substrate. Because of sun-drying for 3-5 days after completion of fermentation, the moisture content of fresh sinki reduced to about 21% in dry sinki which is marketed. This reduced moisture level increases the shelf-life of the product. The proximate composition of radish tap root and sinki showed no difference in the content of protein and fat. While the

pH of radish tap root was slightly acidic (6.72), freshly prepared sinki was distinctly acidic (3.3). Evidently, this was due to increase in acidity (expressed as lactic acid) from 0.04 to 1.28%. On sun-drying, the acidity reduced to 0.72% and the pH increased to 4.4. The marked changes in pH and acidity indicated the need to isolate and identify the dominant acid-producing microorganisms in sinki. Microbial analysis of sinki showed the presence of only lactobacilli because they were Gram positive, catalase negative, microaerophilic and nonsporing rods. The lactobacilli included both homo- and hetero-fermenters. Following the criteria laid down by Kandler and Weiss (1986), the isolates were identified as *Lactobacillus plantarum* Orla-Jensen, *Lactobacillus brevis* Orla-Jensen and *Lactobacillus fermentum* Beijerinck.

The strains belonging to *L. plantarum* did not produce gas from glucose and also did not hydrolyse arginine. In contrast, the strains belonging to *L. brevis* and *L. fermentum* produced gas from glucose and hydrolysed arginine. The strains of *L. brevis* differed from the strains of *L. fermentum* with respect to a number of physiological and biochemical characteristics including hydrolysis of esculin, growth at 15 and 45°C, production of acid from cellobiose, galactose, mannose and sucrose.

Microbial analysis of radish tap root exhibited the presence of *L. plantarum*, *L. brevis* and *L. fermentum*. This substantiates the reports of Mundt and Hammer (1968) about the habitat of lactobacilli. Both *L. plantarum* and *L. brevis* were found in 100% of the market as well as laboratory-made samples of sinki. However, in no case, *L. fermentum* was recovered. This indicates that *in situ*

L. fermentum, whose population was five times higher than the other two species in tap roots, initiated the fermentation, but soon the flora was overtaken by the other two. The presence of *L. plantarum* and *L. brevis* are reported in several fermented foods such as sauerkraut (Pederson 1930a,b; Pederson and Albury 1954, 1969; Stamer et al. 1971) and kimchi (Kim and Whang 1959; Mheen and Kwon 1979); *L. plantarum* is reported in gundruk (Karki et al. 1983d).

The process parameters of traditional methods for the preparation of sinki were optimized by sensory evaluation. The glass jar with lid was found to be an optimum container during fermentation of radish tap root. This is because a glass jar could be filled up to the brim and pressed further until the interspace between the root pieces and the surface are filled with radish juice and subsequently be covered tightly with a lid to create an anaerobic condition for the fermenting organisms. This proved significantly better ($P < 0.05$) than the conventional earthen jar or the alternative polythene bag. The fermentation at 20°C was too slow for the growth of lactobacilli to make the desirable changes in the substrate. Again, the score of the product prepared at 40°C was significantly less ($P < 0.05$) than that prepared at 20°C. This was due to suppression of growth of lactobacilli because of higher temperature. The temperature of 30°C was optimum for the growth of lactobacilli and produced the best fermentation product. The fermentation time of 7 days at 30°C was not enough to complete the fermentation. Again, under long fermentation time of 20 days, the product was highly acidic in taste and flavour. The

product formed after fermentation of 12 days had the highest score, differing significantly ($P < 0.05$) from the other two treatments.

In the first two days of fermentation, the heterofermentative *Lactobacillus fermentum* predominated, reaching a maximum of about 10^7 cfu/g fresh weight. The rapid growth, production of significant amount ($P < 0.05$) of acid and possible production of gas by *L. fermentum* in the initial stages of fermentation made a favourable oxygen-depleting environment for more acid tolerant lactobacilli to be succeeded. Another heterofermenter, *L. brevis*, which dominated on the 4th day of fermentation, also helped to make the condition favourable for more acid tolerants. Second and fourth days onwards, the respective counts of *L. fermentum* and *L. brevis* started to decline rapidly, while the increase in the population size of the homofermentative *L. plantarum* remained unabated till the end of fermentation when it reached a maximum of about 10^9 cfu/g. The growth of the last succeeded organism and the increase in acidity were almost parallel. Interestingly, *L. fermentum* which dominated during the initial period of fermentation, disappeared at the end. The fermentation was, therefore, initiated by *L. fermentum* followed by *L. brevis* and *L. plantarum*.

The initial appearance of all the three lactobacilli during fermentation of radish tap root was due to their presence on the substrate itself. The observation is in accordance with the involvement of lactics during the preparation of sauerkraut (Pederson and Albury 1969) and kimchi (Mheen and Kwon 1979), excepting that *L. fermentum*, instead of *Leuconostoc mesenteroides* initiated fermentation during sinki preparation. As reported by Frazier and Westhoff (1978), gas-forming lactobacilli produced the

same products as the leuconostocs, by attacking sugar to form lactic acid, acetic acid, ethanol, mannitol, esters and carbon dioxide. The level of protein and fat in sinki was not different from that in its substrate. Moreover, all the three sinki isolates were unable to hydrolyse protein, fat and starch. These findings indicate that none of these three fermentable substrates were utilized by the lactobacilli in sinki. Most likely, the free sugars were fermented to cause high acidity of the product. *Lactobacillus plantarum* utilizes mannitol and thus removes the bitter flavour of mannitol produced by the gas-forming lactobacilli.

The microorganisms involved in sinki preparation are similar to those in gundruk preparation, excepting that in gundruk *Pediococcus pentosaceus*, instead of *L. brevis*, is present.

Sinki prepared under optimized conditions had advantages over the traditional methods. Glass jar, as a fermenting container, had packing adequacy, condition of the product inside the jar could be seen from outside, less breakage during packing, more hygienic and could be covered tightly with lid to create an anaerobic condition for the growth of lactobacilli. The fermentation time, one month-long in traditional methods, could be made shorter by fermenting radish tap roots in glass jars at a constant temperature of 30°C.

5.3. MESU

Mesu, a fermented pickle of young bamboo shoot, is similar to naw-mai-dong of Thailand. Mesu differs from the latter in being non-salted and having shorter fermentation time.

The proximate composition indicated no difference between unfermented and fermented bamboo shoot, excepting pH and acidity. The mean moisture, protein and fat contents of fresh mesu were about 94.1%, 17.1% (dry matter basis) and 2.4% (dry matter basis), respectively. The fresh bamboo shoot had mean pH and titratable acidity of 6.35 and 0.04%, respectively, whereas mesu had the mean pH and titratable acidity of 4.04 and 0.83%, respectively.

The pronounced change in pH and acidity indicated the need of isolating and identifying the dominant acid-producing microorganisms in mesu. The microbial analysis of mesu showed the presence of lactobacilli and pediococci. Following the criteria laid down by Kandler and Weiss (1986), the two species of lactobacilli were identified as *Lactobacillus plantarum* Orla-Jensen and *Lactobacillus brevis* Orla-Jensen. The strains having tetrads of coccal cells were identified as *Pediococcus pentosaceus* Mees in accordance with the criteria laid down by Garvie (1986b).

The strains belonging to *L. plantarum* did not produce gas from glucose and also did not hydrolyse arginine. On the contrary, the strains belonging to *L. brevis* produced gas from glucose and hydrolysed arginine.

Microbial analysis of young bamboo shoot exhibited the presence of *L. plantarum*, *L. brevis* and *P. pentosaceus*. This finding supports the report of Garvie (1986b) about the occurrence of pediococci along with lactobacilli in plants. In mesu, *L. plantarum*, with 100% prevalence, was the predominant (ca 10^8 cfu/g fresh weight) microorganism. Although *L. brevis* also was prevalent in 100% of the samples, its count in mesu was about 10^5 times less

than that of *L. plantarum*. The load of *P. pentosaceus*, prevalent in 40-50% of the samples, was minimum (ca 25 cfu/g fresh weight).

The process parameters of traditional methods for the preparation of mesu were optimized. As in sinki, the glass jar with lid and 30°C were determined as the optimum container and the optimum temperature, respectively for fermentation of bamboo shoot. The fermentation time of 10 days was determined as the optimum at a temperature of 30°C which was the optimum temperature for the growth of the lactic isolates. Under these conditions, the product had a desirable soury taste and acidic with a typical mesu flavour.

The successional study revealed that the appearance of *L. plantarum*, *L. brevis* as well as *P. pentosaceus* even at the onset of fermentation, because of their presence in bamboo shoot. The order of changes in microflora during fermentation of bamboo shoot was similar to that of radish tap root in sinki, excepting that in mesu the homofermentative *P. pentosaceus*, instead of the heterofermentative *L. fermentum*, was present. In the first two days, *P. pentosaceus* predominated, reaching a maximum of about 10^7 cfu/g fresh weight. The load of the heterofermentative *L. brevis* reached its peak on the 4th day. Rapid growth, production of significant amount ($P < 0.05$) of acid by these two lactics and the possible production of gas by the latter created an oxygen-depleting condition, suitable for the last successor, *L. plantarum*. After reaching at their peaks, the counts of *P. pentosaceus* and *L. brevis* declined rapidly till the end of fermentation when there was predominance of *L. plantarum*. During fermentation, the decrease in pH and the increase in acidity (expressed as lactic acid) were significant ($P < 0.05$) at every 2 days intervals till the 8th day of

fermentation, after which the changes were not significant ($P < 0.05$).

In naw-mai-dong, *Pediococcus cerevisiae* predominates during the initial stages of fermentation (Dhavises 1972). In contrast to the succession of microflora in mesu, in naw-mai-dong the order is *Pediococcus*, *L. plantarum* and *L. brevis*.

5.4. MURCHA

Unlike other starter cultures such as ragi, bubod, Chinese yeast and loogpang, murcha is usually prepared by wrapping the flattened cakes in fern fronds (commonly *Athyrium* sp.) with the fertile side (bearing sori) touching the cakes. This may probably be due to non-availability of paddy-straw in high altitude areas, and abundance of ferns. Probably, germination of spores in sori helps to maintain the rising temperature of the fermenting mass in cold climates. Other steps of preparation of murcha are similar to the steps of other starter cultures. Murcha is mainly used for fermenting finger millet (*Eleusine coracana* Gaertn.) seeds. Other substrates such as rice, maize, wheat, bajra, sweet potato, ginger or even *Rhododendron* flower petals are also used depending on their availability.

Murcha contains only about 13% moisture. This low level was due to sun-drying after the fermentation. The acidic nature (pH 5.16) of murcha was due to the presence of high population of lactic acid bacteria.

The microbial analysis of murcha collected from these regions showed the prevalence of lactic acid bacteria, yeasts and moulds in all market samples. Only one type of lactic acid bacteria

represented by *Pediococcus pentosaceus* was found. The inability of utilizing starch by *P. pentosaceus* indicates that they are not significant contributors to the breakdown of finger millet or other starchy substrate during preparation of murcha itself or any beverage. Their role is likely to give flavour to the product. The presence of *P. pentosaceus* has been reported in murcha of Nepal (Hesseltine and Ray 1988), and in ragi (Toyota and Kozaki 1978; Hadisepoetro *et al.* 1979; Hesseltine and Ray 1988).

Two types of yeasts were isolated from murcha samples of these regions. Among them, *Saccharomycopsis fibuligera* occurring at a level of $4-68 \times 10^7$ cfu/g was the predominant yeast in murcha. The population of the other yeast, *Pichia anomala* was about ten times less than that of *S. fibuligera*. *Saccharomycopsis fibuligera* represents the only starch-degrading yeast in murcha. The role of *S. fibuligera* as a starch-degrader in starter cultures has been illustrated by Hesseltine and Kurtzman (1990). Batra and Millner (1974, 1976) and Batra (1981, 1986) reported the presence of *S. fibuligera* and *P. anomala* in murcha collected from Darjeeling, Kalimpong, and Rhenock of Sikkim. *Saccharomycopsis fibuligera* has been reported in murcha of Nepal (Hesseltine and Kurtzman 1990), in ragi (Dwidjoseputro and Wolf 1970; Hesseltine and Kurtzman 1990), in Chinese yeast and bubod (Hesseltine and Kurtzman 1990).

The moulds occurring at a level of 10^7 cfu/g fresh weight in the murcha of Darjeeling hills and Sikkim were restricted to *Mucor circinelloides*. In murcha of Nepal, both *Rhizopus* and *Mucor* are present (Hesseltine 1983b; Hesseltine *et al.* 1985, 1988). However, *Rhizopus* strain could not be recovered in this investigation from the murchas of Darjeeling hills and Sikkim. But, Batra and Millner

(1974, 1976) reported the presence of *R. arrhizus*, *M. fragilis* and *M. rouxianus* in murcha collected from Darjeeling, Kalimpong and Rhenock. *Rhizopus* and *Mucor* have also been reported in other similar rice-based cultures such as ragi and Chinese yeast (Hesseltine et al. 1988), bubod (Tanimura et al. 1977) and loogpang (Pichyangkura and Kulprecha 1977). Although present in all rice-based starter cultures, *Amylomyces* was absent in murcha, because during most of the year the temperature is too low in hilly regions and thus affects the growth of this mould (Hesseltine et al. 1988).

When one thinks over any new technology particularly for a developing country, an important consideration must be labour input and energy requirements. In this respect, all these fermentations appear to be very appropriate processes. The labour input is not excessive and the work can be and is usually done by elderly people of the house. The only energy source needed in many of these foods is the sun.

Traditional processing methods are surely not ideal and there is ample scope for improvements. This is shown in the processes just described. But, nevertheless, as those techniques developed through a number of generations, they take into account all the constraints given by the environment and the culture. Hence, they are well worth a scientific study. They can reveal the value of traditional techniques and people can be reassured about the worth of their knowledge.

6. SUMMARY

The people of Darjeeling hills and Sikkim consume a variety of traditional fermented foods including kinema (soya bean product), masayura (black gram product), gundruk (leafy vegetable product), sinki (radish tap root product), mesu (young bamboo shoot product), khalpi (cucumber pickle), shel roti (rice preparation), dahi (dairy product), kachcha churpi (dairy product), churpi (dairy product), dudh churpi (dairy product), sukako masu (meat product), murcha (starter culture, rice flour product), jnard (beverage) and raksi (beverage).

Kinema, a meat substitute to the majority of the people of these regions, is fried and made to a thick curry to use as a side dish of rice. Masayura, a ball-like or conical, hollow, black gram product, is used as a spicy condiment. Gundruk and sinki are dried sour vegetable products, eaten as soup or pickle. Mesu and khalpi are fermented pickles. Shel roti is a deep fried, ring-shaped confectionary bread, produced from fermented rice batter. Dahi is an acidic savory. Kachcha churpi, a soft mass product of cow's milk, is used as a condiment. Churpi and dudh churpi are hard products, prepared from cow's or yak's milk and used as masticatory. Sukako masu, prepared by smoking the strips of mutton, pork, beef or yak meat, is eaten as curry. Murcha, a spherical, flattened, solid cake, is a starter culture prepared from rice flour, wild herbs and spices, used to ferment finger millet seeds to produce a popular, mild alcoholic beverage known as jnard. Other starchy materials are also used to prepare jnard. Raksi is a distilled part of jnard, usually prepared from rice. All these foods are very important in that they are socio-culturally related. Majority

of the people of these regions consume all the foods mentioned above excepting mesu, khalpi and masayura, the consumption of which is confined to a few pockets of the hills.

Most of these fermented foods have not previously been investigated. In this report, the traditional methods of their preparation, mode of consumption and ethnic importance have been documented. Kinema, sinki, mesu and murcha were selected for microbial and biochemical studies.

Kinema contains a high amount of moisture. The moisture content of about 11% in raw soya beans increased to about 62% in kinema. This is a protein-rich product containing about 48% (on dry matter basis) protein. While the fat content in raw beans was about 22%, the same in kinema was about 17%. Since ash is usually added during preparation of kinema for marketing, the ash content of market samples showed a higher value, compared to that of the laboratory-made samples of kinema. Much higher values of pH, titratable acidity and free fatty acidity were observed in kinema than their corresponding values in soya beans. The energy values of soya beans and kinema were nearly the same (ca 2 MJ per 100 g dry matter).

A total of 502 bacterial and 198 yeast strains were isolated from 50 samples of Kinema. All the sporeforming bacteria were tentatively identified as *Bacillus subtilis*, and the asporogenous cocci were identified as *Enterococcus faecium*. The yeasts were identified as *Candida parapsilosis* and *Geotrichum candidum*. The load of *B. subtilis*, the only organism recovered in raw soya beans at 8×10^5 cfu/g, was $3-5 \times 10^8$ cfu/g fresh kinema with 100%.

prevalence in both the substrate and the fermented product.

Enterococcus faecium, with a load of $5-9 \times 10^7$ cfu/g, occurred in 100% samples of kinema. The population of *C. parapsilosis* with 50-80% prevalence was $0.3-9 \times 10^4$ cfu/g, and that of *G. candidum*, recovered from 40-50% of the market samples only, was $0.8-4 \times 10^4$ cfu/g.

The traditional process parameters for the preparation of kinema were optimized at 10 min time for cooking soya beans at 0.7 kg/cm² steam pressure, and fermenting beans in perforated polythene bag, and at 37°C for 48 h. Studies on microbial changes during soya bean fermentation indicated *Bacillus subtilis* as the predominant microorganism which increased significantly ($P < 0.05$) at every 8 h intervals till the end of fermentation. The population of *Enterococcus faecium* also increased at an approximately same rate as of *B. subtilis*. The load of *Candida parapsilosis*, although much less compared to the bacterial load, increased significantly ($P < 0.05$) at every 8 h intervals till 32 h of fermentation. In laboratory preparations, *Geotrichum candidum* could not be detected. The biochemical changes during fermentation of soya beans revealed the initial decline in pH from 6.94 to 6.64 at 16 h after which there was a sharp increase in pH up to 8.51 at 40 h. The titratable and free fatty acid contents increased significantly ($P < 0.05$) at every 8 h intervals during the entire course of fermentation. The moisture content remained relatively constant at about 62%. At the end of fermentation, the total nitrogen content increased significantly ($P < 0.05$) over the substrate at 0 h of fermentation. Due to proteolytic activities of *B. subtilis*, there were remarkable

changes in protein, non-protein and soluble nitrogen during the fermentation. While the protein nitrogen declined, the non-protein and soluble nitrogen contents increased significantly ($P < 0.05$) at almost every 8 h intervals till the end of fermentation.

Bacillus subtilis DK-W1, isolated from kinema of Darjeeling market, was selected as the best proven strain for production of kinema. Attempts were made to improve the general acceptability of kinema by inoculating sterile beans with that strain and fermenting at 45°C for 18 h, optimized earlier. The studies revealed that *B. subtilis* DK-W1 could effectively be used for desirable fermentation within a much shorter period and with excellent product development comparable to the natural fermentation of kinema. The changes in proximate composition of the fermenting beans were similar in monoculture and natural fermentations.

The market sinki had about 21% moisture, while the freshly prepared laboratory-made samples had 93.5% moisture. Regarding the contents of protein, fat and ash, no change was observed between raw radish tap root and sinki. There was a remarkable decrease in pH and increase in acidity in sinki from its substrate. A total of 453 strains of lactic acid bacteria were isolated from 40 samples of sinki. Two species of *Lactobacillus*, *L. plantarum* and *L. brevis* were isolated from sinki. In addition to these two, *L. fermentum* was present ⁱⁿ the substrate. In sinki, *L. plantarum* was dominant (6×10^8 cfu/g) followed by *L. brevis* ($6-7 \times 10^3$ cfu/g) with their prevalence in 100% of the samples analysed.

The traditional process parameters for the production of sinki were optimized at fermenting radish tap root in glass jar at 30°C for 12 days. The fermentation was initiated by heterofermentative *Lactobacillus fermentum*, followed by another heterofermentative *L. brevis*, both dominated in the early stages of fermentation, and succeeded later on by more acid-producing homofermentative *L. plantarum* till the end of fermentation, when *L. fermentum*, which initiated the process, disappeared. During fermentation, the drop in pH and rise in titratable acidity were 6.72 to 3.30 and 0.04 to 1.28%, respectively. The moisture and total nitrogen contents remained relatively constant throughout fermentation.

The contents of moisture, protein, fat and ash were same in both bamboo shoot and mesu. The remarkable decrease in pH and increase in acidity in mesu over its substrate were observed. A total of 327 strains of lactic acid bacteria representing *Lactobacillus plantarum*, *L. brevis* and *Pediococcus pentosaceus* were isolated from 30 samples of mesu. All these lactics were present in 100% samples of the raw bamboo shoots. In mesu, *L. plantarum* was dominant (3×10^8 cfu/g) followed by *L. brevis* ($4-5 \times 10^3$ cfu/g) with 100% prevalence in both of them. *Pediococcus pentosaceus* was least populated (20-30 cfu/g), and recovered from 40-50% of the market samples.

The traditional process parameters for the production of mesu were optimized at fermenting bamboo shoots in glass jar at 30°C for 10 days. During fermentation, initially, the homofermentative *P. pentosaceus* comprised the most dominant microflora. Its

dominance was soon overtaken by the heterofermentative *Lactobacillus brevis* which attained its peak at the 4th day of fermentation.

This was finally succeeded by the more acid-producing homofermentative *L. plantarum* which became the dominant organism at the end. The fermentation caused the decline in pH from 6.35 to 3.84 and increase in acidity from 0.04 to 0.95%. Moisture and total nitrogen contents remained the same.

Murcha contains 13% moisture and 0.7% ash (dry matter basis), having pH 5.1-5.2. A total of 194 bacterial, 190 yeast and 58 mould strains were isolated from 30 market samples of murcha. The bacterial strains were identified as *Pediococcus pentosaceus*, the yeasts as *Saccharomycopsis fibuligera* and *Pichia anomala*, and the moulds as *Mucor circinelloides*. The samples contained (cfu/g fresh weight): $1.9-2.1 \times 10^8$ *P. pentosaceus*, $3.6-3.9 \times 10^8$ *S. fibuligera*, $2.8-3.0 \times 10^7$ *P. anomala* and $2.0-2.3 \times 10^7$ *M. circinelloides*. All these organisms were present in 100% of the samples analysed.

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Traditional Fermented Foods and Beverages of Darjeeling and Sikkim—a Review

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ABSTRACT

The various ethnic groups of the Darjeeling district of West Bengal and Sikkim consume a variety of fermented foods including kinema (based on soya beans), gundruk (Brassica campestris leaves), sinki (radish, Raphanus sativus), mesu (bamboo shoots), churpi (milk), shel roti (rice preparation) and jnards (beers). These have not previously been investigated, and their method of preparation and consumption are reported here. The flora of murcha, the starter culture of jnards, contains mainly Pediococcus, yeasts belonging to the genera Saccharomycopsis, Pichia and Saccharomyces and the moulds Rhizopus and Mucor.

Key words: Kinema, gundruk, sinki, mesu, churpi, shel roti, jnard, murcha, fermentation, soya bean, *Brassica campestris*, *Raphanus sativus*, bamboo shoots, milk, rice, yeasts.

1 INTRODUCTION

A food is considered fermented when one or more of its constituents has been acted upon by microorganisms to produce a considerably altered final product acceptable for human use (van Veen 1957). Fermented foods are important components of the

diet as staples, adjuncts to staples, condiments and beverages. There is a wide variety of fermenting substrates, such as cereals, pulses, soya beans, flowers, milk, fish, meat, etc. Fermentation increases the digestibility of pulses (van Veen and Schaefer 1950). In some fermented products containing spices and salt, the keeping quality is considerably enhanced and shelf life is prolonged. Other attributes are improved flavour and appearance and reduced cooking time. Fermented foods provide variety in the diet (Batra and Millner 1976). Increased levels of water-soluble vitamins have been reported in tempeh (Wang and Hesselstine 1966; Rajalakshmi and Vanaja 1967; van Veen and Steinkraus 1970).

About 70% of the inhabitants of the Darjeeling district of the state of West Bengal and about 90% in the state of Sikkim (a total of 1.15 million people) traditionally consume large quantities of fermented foods and beverages. Some ethnic groups are economically dependent upon these local products. The fermentation techniques are passed on as trade secrets in the families of certain communities, a practice protected by tradition. The common fermented foods and beverages of the region include kinema, gundruk, sinki, mesu, churpi, shel roti and a variety of jnards. Although there is information on the availability of kinema from Darjeeling, Sikkim and Nepal (Batra and Millner 1976), gundruk and sinki from Nepal (Karki *et al* 1983), and thumba from Darjeeling, Sikkim and Nepal (Batra and Millner 1976; Hesselstine 1979), in the cases of mesu, churpi, shel roti and other kinds of jnard there is no record.

The aim of this review is to assess the ethnic value, nature, method of preparation and mode of consumption of these fermented products, much of which information has not hitherto been generally available.

2 FOODS

2.1 Kinema

Although traditionally used by the Nepalese, kinema is now popular among the Lepchas and Sikkimese who call it respectively 'satlyangser' and 'bhari'. The kinema production process is shown in Fig 1. Soya beans (*Glycine max* (L) Merr) are washed, soaked in water overnight, cooked by boiling and cooled to room temperature. They are then crushed lightly with a wooden ladle to split the kernels. A small amount of firewood ash is added and blended with the whole soya bean grits which are traditionally wrapped with banana (*Musa paradisiaca* L) or *Leucoscepterum canum* Smith leaves; polythene bags are also sometimes used. The wrapped mass is covered with sackcloth and kept in a warm place, usually above an earthen oven in the kitchen, for 1-2 days during summer or 2-3 days in winter. The desired state of fermentation is indicated by the formation of mucilage and an unpleasant ammoniacal aroma. Kalimpong kinema has a darker, brown colour but is less mucilaginous than the kinema from elsewhere. The product is very similar to Indonesian tempeh and Japanese natto.

Kinema (Fig 2a) is used to give a pleasant, nut-like flavour to curry. It is also dried, fried in edible oil and mixed with salt, onion and chillies to produce pickle.

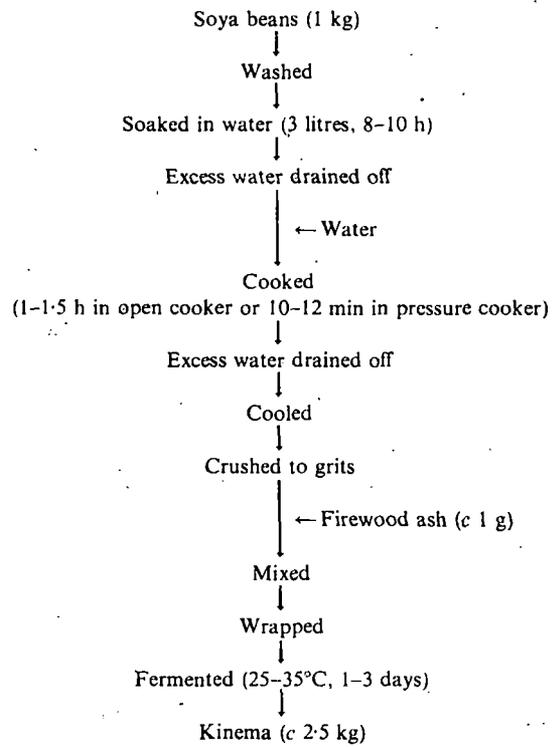


Fig 1. Flow sheet of kinema production.



Fig 2. Kinema (a), gundruk (b) and sinki (c), as sold in markets.

Fresh kinema keeps for a maximum of one week. The shelf life is often lengthened to one month by drying in the sun or by keeping on earthen ovens in kitchens.

2.2 Gundruk

The word 'gundruk' is derived from the Newari word 'gundru' (the Newaris being one of the ethnic groups of the Nepalese). It is traditionally used by the Nepalese, but is now popular among all the ethnic groups of Darjeeling and Sikkim. The fermenting substrate for gundruk is usually 'rayo' (*Brassica campestris* L var *cumifolia* Roxb) leaves. Other leaves such as radish (*Raphanus sativus* L), shimrayo (*Cardamine hirsuta* L var *sylvatica* Link), cauliflower (*Brassica oleracea* L var *botrytis*-L), etc are also used. Gundruk (Fig 2b) is usually prepared during the months of December to February when the weather is less humid and there is an ample supply of the vegetables. Prepared in other seasons, particularly during the monsoon, it is said to decay rapidly and to have an unpleasant flavour. The preparation (Fig 3) takes about a month. Leaves are dried in the sun (1–2 days depending on the weather). The dried leaves, after a mild crushing, are soaked briefly in hot water and, hand-pressed in a perforated tin or earthen jar with a heavy article such as a large stone to remove surplus water. They are then kept in a warm and dry place for 15–22 days. In the village process a hole of diameter and depth of ~1 m is dug in the ground and dried by fire, and a 30-cm layer of banana or bamboo leaves is placed in the bottom; the dried crushed leaves of the vegetables to be fermented are placed above this layer and covered with a further layer of banana or bamboo leaves. Heavy stones are added to compress the substrate. The holes are sometimes finally covered with a layer of cow dung. The leaves are allowed to ferment *in situ* until a fermentation odour develops (15–22 days). The gundruk is taken out and dried in the sun for 2–4 days. It has a shelflife of about one year.

A very similar product, known as 'pani [water] gundruk', is commonly prepared and consumed in the Pedong area (in the Kalimpong subdivision of the Darjeeling district). Rayo leaves are sun dried for 2–3 days, crushed lightly, soaked in hot

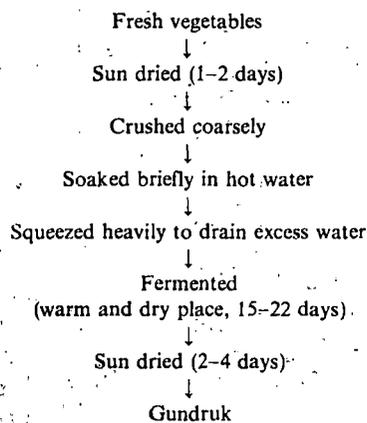


Fig 3. Flow sheet for gundruk production.

water, drained and kept in a vat containing warm water (30–35°C) for only 3 days. They are then squeezed and sun dried for about 7 days. Inhabitants of this region usually prefer pani gundruk to common gundruk for its sour taste, easier preparation and greater resistance to spoilage.

Gundruk is typically used as a base for soup and as a pickle. The soup is made by soaking gundruk in water for 2 min, squeezing out the liquid and frying the residue along with tomato, onion and dried chilli powder. The fried mixture is then boiled in rice water, or in water supplemented with wheat flour, and the soup is served hot as an appetiser. The pickle is prepared by soaking gundruk in water, squeezing and mixing with salt, oil, onion and green chillies.

2.3 Sinki

Like kinema and gundruk, sinki (Fig 2c) was formerly confined to Nepali communities but is now consumed by all the ethnic people of this region. 'Sinki' is a Newari word. The method of preparation is similar to that of gundruk except that the substrate is the tap roots of radish (*Raphanus sativus* L.) and the fermentation takes 30–40 days. The season of its preparation, its mode of consumption and its shelflife are similar to those of gundruk.

2.4 Mesu

This, originally confined to the Nepalese, has become a common food used by all the people of this region. The Lepchas call it 'sitit'. Young edible bamboo ('choya bans' or 'tama', *Dendrocalamus hamiltonii* Nees, or 'karati bans', *Bambusa tulda* Roxb) shoots are finely chopped (1–1.5 cm × 0.3–0.7 cm) and traditionally put into a 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 or 8 days. Mesu is usually prepared in the months of June to September. It has a sour taste and a strong ammoniacal odour. A very common pickle is produced by mixing mesu with salt and green chillies. It is also used for preparing curry by frying and mixing with cooked meats. The naw-mai-dong of Thailand is similar.

2.5 Churpi

This fermented cow's milk product is commonly used by the Tibetans inhabiting this region. Two different kinds of churpi, soft and hard, are available. The soft type is prepared in both hilly and terai areas (the plain land of the Darjeeling district which is at the foot of the hills) and the hard type is restricted to the high altitudes (1300–4000 m) of the Darjeeling district and North and East Sikkim.

Soft churpi (called 'kachcha churpi' by the Nepalis and 'chuiw' by the Lepchas) is prepared (Fig 4) from cow's milk. The milk, boiled or unboiled, is kept in a wooden vat at room temperature for 24 h. Often the cream is allowed to separate and the milk is curdled by boiling. The casein is wrapped tightly in a piece of muslin cloth and allowed to drain for 3–5 h. The churpi is consumed as a condiment by mixing with sliced radish or cucumber; it is also mixed with meats, vegetables and spices to prepare curry.

Hard churpi ('churpi' to the Nepalis; 'chura' to the Sikkimese; 'khamum' to the

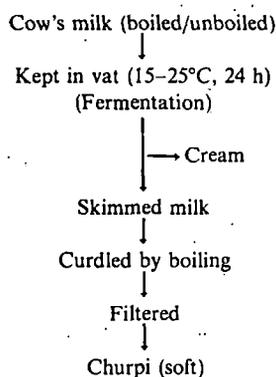


Fig 4. Flow sheet for soft churpi production.

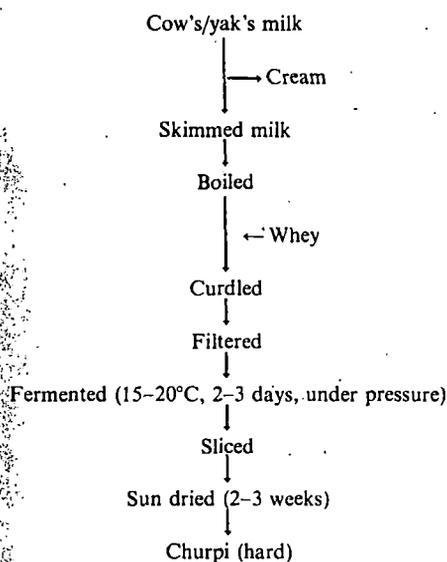


Fig 5. Flow sheet for hard churpi production.

Lepchas) is prepared (Fig 5) from cow's or yak's milk. The cream is separated from milk by centrifugation and the skimmed milk is boiled and curdled by adding whey. After filtration, the casein is wrapped tightly with a cloth and cured at room temperature (15–20°C) for 2–3 days under pressure of about 0.25 kg/cm² made with the aid of heavy stones. The cheeses are sliced and allowed to sun dry for 2–3 weeks. This type of churpi becomes very hard and, having a low moisture content, can be stored for a number of years. Churpi is sweet in taste and is used in much the same way as chewing gum. Chu-ra, a kind of cheese similar to hard churpi, has been reported to be commonplace in Tibet, Nepal and north-east India (Batra and Millner 1976).

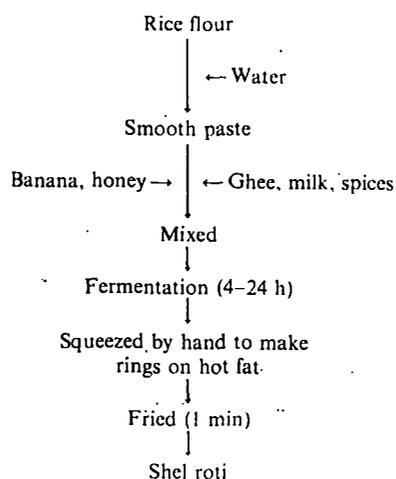


Fig 6. Flow sheet for shel roti production.



Fig 7. Shel roti as marketed.

2.6 Shel roti

These are ring-shaped fried foods eaten traditionally by the Nepalese of hilly regions. They are usually prepared (Fig 6) by mixing rice-flour paste (rice 1 kg and water 1 litre) with banana (two small pieces), honey (100 g), ghee (100 g) and some spices. Sometimes, instead of banana and honey, sodium bicarbonate (3 g) and sugar (200 g) are added. The well mixed batter is allowed to ferment for between 4 h (during summer) and 24 h (during winter). The leavened batter is squeezed by hand and deposited as continuous rings into hot fat. These rings (Fig 7) are fried and served hot.

3 BEVERAGES

3.1 Jnard

Jnard is a common drink in the region, and is traditionally prepared by almost all Nepalese and Tibetans. This slightly acidic, alcoholic beverage is now prepared by small cottage industries. The word 'jnard', derived from the Mangaranti language (the Mangarantis being one of the ethnic groups of the Nepalese) is known by many synonyms ('chiang' by the Tibetans, 'chii' by the Rong, 'toongba' by the Nepalese, who take jnard in a bamboo vessel; the word 'toongba' actually means the bamboo vessel in which the jnard is consumed). Jnard is the fermentation product of finger millet (*Eleusine coracana* Gaertn), locally known as 'kodo' or 'marua', and is commonly cultivated in the Kalimpong area of the Darjeeling district and at lower altitudes (up to 1300 m) in Sikkim. Finger millet seeds are sometimes supplemented with a small amount of wheat or corn grains. The seeds are boiled in an open cooker for about 30 min and spread on leaves, preferably of banana plants. 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. They are then usually placed in an earthen pot and covered with leaves followed by cow dung. In urban areas the seeds are allowed to ferment in a polythene bag. If air is allowed access to the fermentation, and sometimes even when the pots are kept airtight, the product becomes sour. After fermentation the seeds are kneaded to remove the seed coats. The grits are placed in a bamboo vessel (toongba) and water is added. After ~10 min the beverage is ready to drink (normally through a bamboo straw). This liquor is believed to be a good tonic, especially for post-natal women. Thumba, a very similar kind of beverage, has been reported to be produced in India (Batra and Millner 1976, Hesseltine 1979).

Although the term 'jnard' is commonly used for finger millet beers, beers from other substrates such as maize, rice, wheat, bajra, sweet potato, ginger, *Rhododendron* flowers, etc are also called jnards, the name of the beer deriving from the raw material used for the fermentation eg 'makai jnard' (maize beer), 'vate jnard' (rice beer). Their modes of production and consumption are similar to those of toongba. However, the period of fermentation differs from substrate to substrate.

4 STARTER CULTURE

Murcha is not a food but a starter culture used for several beverage fermentations commonly consumed locally. 'Murcha' is a Nepali word; the Lepchas use the word 'thamik' and the Limbu (one of the ethnic groups of the Nepalese) 'khesung'. Trade in murcha is protected as a hereditary right of certain castes of the Nepalese (particularly Limbu and Rai) and the Lepchas. Figure 8 illustrates the sequential addition of ingredients. Rice (glutinous and non-scented), soaked in water overnight (10–12 h), is placed in a large wooden mortar. A few pieces of the root of 'sweto-chitu' (*Plumbago zeylanica* L), commonly used by the Lepchas of Bong Busty, Kalimpong), a few leaves of 'vimsen pathe' (*Buddleja asiatica* Lour), and certain spices such as ginger and chilli, are blended using a heavy wooden

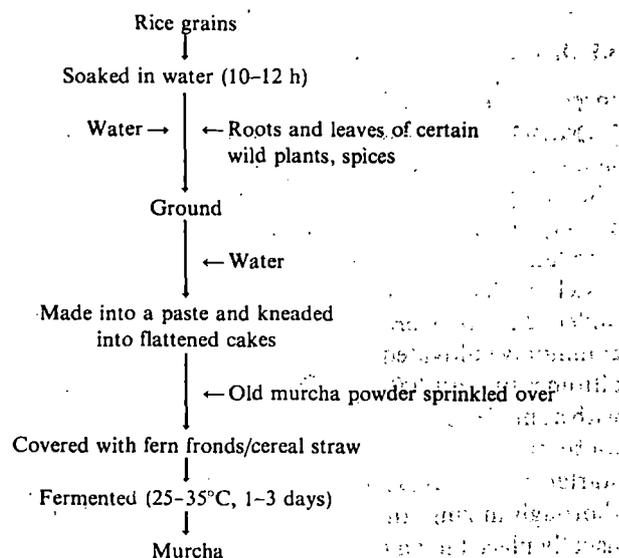


Fig 8. Flow sheet for murcha production.

pestle. Water is added to make a thick paste which is kneaded to give flattened cakes 2-5 cm in diameter. Old murcha is powdered and sprinkled over the new cakes which are then wrapped in fern fronds with the fertile side (bearing the sori) touching the cake. The cakes are placed on a straw-covered bamboo floor above kitchen ovens and covered successively with fresh fronds, dried fronds, straw and finally sackcloth. They take 1-3 days to dry at 25-35°C. This method is practised by the Rai and Limbu communities. The Lepchas cover the cakes with rice straw instead of fern fronds. After air drying, the cakes are dried in the sun, and dry adhering fronds are removed. Murchas remain active for several months at room temperature. In markets, murchas (Fig 9) are sold by the piece.

The microflora of murcha was studied using nine samples from Nepal and India kindly supplied by Dr T Karki, Kathmandu, Nepal. Dilution plates on appropriate nutrient agars were made, into which inhibitors were incorporated. The murcha samples showed similar populations of bacteria, yeasts and moulds. In each group only a few species regularly occurred in large numbers. The bacterial count ranged from 5.5×10^5 to 2.1×10^8 . Most of the isolates belonged to *Pediococcus pentosaceus* Mees. Sixteen isolates were examined and found to vary in their ability to grow at 45°C. They grew well aerobically at both pH 5 and pH 9 but failed to grow in 4% NaCl. In general they failed to utilise mannitol, sorbitol, glycerol, sorbose, lactose, sucrose, arginine and starch but utilised trehalose. There were some exceptions with mannitol and sucrose, and fermentation studies with these species on rice as substrate indicated that they were not significant contributors to the breakdown of cassava and rice starch. Their role may be to give flavour to the product. Similar results were obtained with products from Indonesia, the Philippines and China.

The yeast population in the nine samples was very high, ranging from 5.4×10^6 to



Fig 9. Murcha cakes as marketed.

6.1×10^8 , and the mould count was 1.5×10^6 to 2.8×10^8 . As with the bacteria, the variety of yeast and mould species was very restricted. Thus, of the 26 yeast strains, 19 were *Saccharomycopsis fibuligera* (Linder) Klockner, one was of *Pichia anomala* (Hansen) Kurtzman and another was a *Saccharomyces* sp; the other five were unknown. *S. fibuligera* utilises starch, grows well on cellobiose and has a fermentation temperature of 35°C. It requires pyridoxine for growth and fails to grow on arabitol and glucuronate.

The moulds in the murcha starters are restricted to species of *Rhizopus* and *Mucor*, all members of the Mucorales. The *Mucor* species are mostly *M. circinelloides* van Tieghem group and *M. indicus* Lendner. Unlike most fungi, the murcha strains of *Rhizopus* and *Mucor* will grow under strict anaerobic conditions as long as CO₂ is supplied (Hesseltine *et al* 1985). This accounts for the fact that they are the only moulds that occur in murcha. Murcha, unlike ragi (a similar product), does not have *Amylomyces* as a part of the regular flora. Interestingly, the moulds and yeasts survive for years in a dry state at 4–5°C. For example, plating out of the four-year-old murcha samples revealed both *Rhizopus* and *Mucor* to be alive in all samples and able to grow vigorously. The *Rhizopus* species appear to belong to the *R. chinensis* Saito and *R. oryzae* Went *et P* Geerlings groups.

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