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 easiliy 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 Hesseltine (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; Hesseltine 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 Hesseltine 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/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 beacause 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 cid 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 x 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.