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Native microorganisms in the fermentation of *kinema*

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***Kinema* is a naturally fermented indigenous soybean food, common to the eastern himalayan regions of India, Nepal and Bhutan. Microbial diversity in *kinema* was assessed to establish the source of inocula during spontaneous fermentation. Microorganisms associated with *kinema* were present in or on the ingredient, equipment, ash and wrapping materials, which also contributed significant genetic resources. Species of *Bacillus, Enterococcus, Geotrichum, Candida* were recovered.**

Key words: *Kinema*, microbial diversity, spontaneous fermentation, *Bacillus, Enterococcus*

Kinema is a popular fermented soybean-based, gray tan coloured, slightly alkaline, sticky food having ammonical odour, which serves as cheap source of high protein food in a local diet. Preparation and consumption of *kinema* reflect deep rooted food culture of ethnic people of the eastern himalayan regions of Darjeeling hills and Sikkim in India, Nepal and Bhutan. The preparation of *kinema* is exclusively practiced by mountain women using their indigenous knowledge. Overnight-soaked soybeans are cooked until they can be pressed easily, then cracked lightly in wooden mortar (locally called *okhli*) and pestle (locally called *muslo*). Grits are placed in a bamboo basket, lined with locally grown fresh fern (or sometime, fig) leaves, about 1% of fire-wood ash is dusted, covered with a jute-bag and left to ferment naturally at ambient temperatures (25 - 40° C) for 2 - 3 d above earthen-oven in kitchen (1). *Kinema* is similar to *natto* of Japan, *chungkok-jang* of Korea, *thua-nao* of Thailand, *douchi* of China, *pe-poke* of Myanmar; *hawaijar* of Manipur, *aakhoni* of Nagaland, *turangbai* of Meghalaya and *bekanthu* of Mizoram in northeast India (2, 3). Microbiota associated with natural fermentation of *kinema* has not been assessed. We examined the materials and equipment involved in *kinema* preparation, such as soybean, wooden mortar and pestle, fire-wood ash, fresh leaves used as wrapping materials so as to establish the source of microorganisms associated during natural fermentation of *kinema*.

Materials and Methods

Sample collection: Local variety of soybean having Yellow-seed [*Glycine max* (L.) Merrill] as well as *kinema* samples were purchased from Gangtok market, aseptically kept in ice-box and transported to laboratory for analyses. Fresh leaves of fern [locally called *pire union*, {*Glaphylopteriopsis erubescens* (Well ex. Hook.) Ching}] and fig plant (locally called *nevara*, *Ficus hookeriana* Corner.) and wood-ash were collected from villages in Sikkim, where *kinema* is made.

Microbial analysis: Ten g of well-mixed sample was blended in 90 mL sterile physiological saline in a stomacher lab-blender 400 (Seward, UK) for 10 min. For aerobic endospore-counts, 1 mL dilution was mixed with 9 mL sterile physiological saline, and heated for 2 min in continuously boiling water (4). Decimal dilution series was prepared in sterile diluent and 1 mL of appropriate diluted suspension was mixed with molten media and poured into plates.

Wooden mortar and pestle used during *kinema* preparation were rinsed with sterile distilled water, which was collected in sterile bottles, and transported immediately to laboratory for microbial analysis using dilution plate method as described above.

Circular discs (0.5 cm diameter) of fresh leaves of fern and fig were cut with flamed sterilised cork borer and washed in sterile physiological saline for isolation of phylloplane microflora (5). One mL of leaf-wash was inoculated into plate containing nutrient agar (HiMedia M001) for enumeration of endospore-forming bacteria and incubated at 37° C for 24 h. Lactic acid bacteria were enumerated on de Man, Rogosa and Sharpe (MRS) agar (HiMedia M641) supplemented with 1% calcium carbonate and incubated anaerobically in an Anaerobic Gas-Pak system (HiMedia LE001) at 30° C for 72 h. Total viable counts were determined using plate count agar (HiMedia M091A) after

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incubating at 30° C for 48 h. Presence of the members of Enterobacteriaceae was tested by using selective violet red bile glucose agar (HiMedia M581) and incubating at 30° C for 48 h. Samples were examined for the presence of yeasts and moulds, using malt extract agar (HiMedia M924) supplemented with 100 mgL^{-1} chloramphenicol and incubating aerobically at 28° C for 72 h. Typical colonies were isolated from different samples and subjected to taxonomical identification.

Phenotypic characterization and identification: Initial characterization of bacterial isolates included colony and cell morphology, Gram staining and catalase reactions. Motility test was observed in a phase contrast microscope (Olympus CH3-BH-PC, Japan) following the method of Harrigan (6). All other phenotypic characterizations of bacterial isolates were carried as per standard procedures (7, 8). Ability of the bacterial isolates to ferment carbohydrates was studied using API 50 CHB and API 20 Strep (bioMérieux, France) system. Endospore-forming bacteria were identified according to the keys based on Claus and Berkeley (9), and Slepecky and Hemphill (10). Lactic acid bacteria were identified following the taxonomic keys laid down in Bergey's Manual of Systematic Bacteriology, (11) and by Wood and Holzapfel (12). Yeast-isolates were identified by the standard morphological and biochemical tests (13).

Enzymatic profiles: The enzymatic profile of bacterial isolates was assayed following the standard method (14) using API zym (bioMérieux, France) galleries based on the manufacturer's colour chart.

Results and Discussion

Microbial load in different sources during spontaneous fermentation of *kinema* is shown in Fig 1. Fifty six strains of rod-shaped, Gram-positive, endospore-forming bacteria were identified as *Bacillus subtilis* (Ehrenberg) Cohn according to the criteria laid down by Claus and Berkeley (9). Following the taxonomical keys (12,15), 30 strains of cocci-shaped, Gram-positive, catalase-negative, non-spore-forming lactic acid bacteria were identified as *Enterococcus faecium* (Orla-Jensen) Schleifer and Klipper-Bälz. Two types of yeasts were recovered and were identified as *Candida parapsilosis* (Ashford) Langeron and Talice and *Geotrichum candidum* Link following the taxonomical keys (13).

Microbial analysis of raw soybeans showed the presence of *Bacillus subtilis* spores. Hesseltine (16) also reported presence of *B. subtilis* on raw soybeans. Besides *B. subtilis*, population of *E. faecium* and yeasts predominantly occurred during soaking of soybeans, indicating their entry through water source. *E. faecium* appears as non-faecal contaminant (17). Mulyowidarso *et al* (18) reported the presence of *E. faecium* during soaking of soybeans in *tempeh* fermentation. Level of Enterobacteriaceae was $<10^4$ cfu g^{-1} in overnight-soaked soybeans. Mortar and pestle also supplemented population of Enterobacteriaceae, which was detected at a level of 10^2 cfu g^{-1}. Cells of microorganisms were killed during cooking of soaked soybeans except heat-resistant spore-formers. *B. subtilis* was found in all sources indicating its main role in fermentation. Source of *E. faecium, C. parapsilosis* and *G. candidum* was mainly from wooden mortar and pestle commonly used to crack cooked soybeans before wrapping during *kinema* fermentation. Usually wooden mortar and pestle are not washed properly by the rural *kinema*-makers after cracking soybeans. This equipment is the main source of inocula for spontaneous fermentation of *kinema*. Phylloplane studies of fresh fern fronds and fig leaves used as wrapping materials also supplement *B. subtilis* and *E. faecium*. Yeasts and moulds were not recovered from these leaves. Singh and Umabati Devi (19) reported the presence of *Bacillus* and *Xanthomonas* spp. in fig leaves, used as wrapping material during *hawaijar* production. The population of yeasts as well of lactic acid bacteria were found to increase remarkably in *kinema*, stored at room temperature after desirable fermentation (Fig 1).

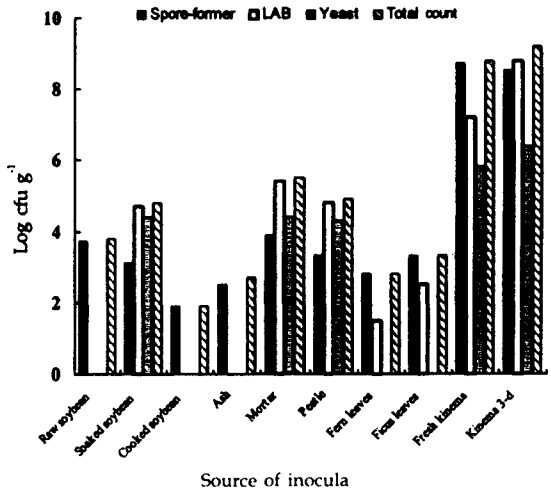

Fig 1. Load of microorganisms associated during spontaneous fermentation of *kinema*. Data represent the means of three replications.

Bacillus-strains produced a wide spectrum of enzymes, whereas strains of *Enterococcus* exhibited

Table 1. Enzymatic profiles of bacterial strains isolated from different sources during spontaneous fermentation of *kinema*

Enzyme	Substrate	Activity (nanomoles)	
		Bacillus subtilis (n = 10)	Enterococcus *faecium* (n = 6)
Phosphatase alkaline	2-Napthyl phosphate	≥ 40	0
Esterase (C4)	2-Napthyl butyrate	5	5
Esterase lipase (C8)	2-Napthyl caprylate	5	5
Lipase (C14)	2-Napthyl myristate	0	0
Leucine arylamidase	L-Leucyl-2-napthylamide	≥ 40	10
Valine arylamidase	L-Valyl-2-napthylamide	0	0
Cystine arylamidase	L-Cystyl-2-napthylamide	0	0
Trypsin	N-Benzoyl-DL-arginine-2-napthylamide	0	0
Chymotrypsin	N-Glutaryl-phenylalanine-2-napthylamide	0	0
Phosphatase acid	2-Napthyl phosphate	> 40	20
Naphthol-AS-BI-phosphohydrolase	Napthol-AS-BI-phosphate	≥ 40	5
α-galactosidase	6-Br-2-napthyl-αD-galactopyranoside	0	0
β-galactosidase	2-Napthyl-βD-galactopyranoside	0	0
β-glucuronidase	Napthol-AS-BI-βD-glucuronide	0	0
α-glucosidase	2-Napthyl-αD-glucopyranoside	5	0
β-glucosidase	6-Br-2-napthyl-βD- glucopyranoside	0	0
N-acetyl-β-glucosaminidase	1-Napthyl-N-acetyl-βD-glucosaminide	0	0
α-mannosidase	6-Br-2-napthyl-αD-mannopyranoside	0	0
α-fucosidase	2-Napthyl-αL-fucopyranoside	0	0

n = Number of strains

less enzymatic activity (Table 1). *Bacillus*-strains showed strong peptidase and phosphatase and relatively week esterase and lipase activities. However, no detectable proteinase activity could be recorded with the method used. Presence of high peptidase (leucine), low esterase-lipase (C4 and C8) and no proteinases (trypsin and chymotrypsin) activities are the desirable traits of *Bacillus* for their use in *kinema* production, probably for typical flavour development.

It is evident that rich microbial diversity in various sources particularly equipment, raw material and leaves harness indigenous microbiota for spontaneous fermentation of *kinema*. The practice of not cleaning the mortar and pestle and using fresh leaves as wrapping materials signify indigenous knowledge of 'microbiology' by rural people, to preserve and supplement microorganisms for spontaneous fermentation of *kinema* without using starter cultures.

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