DISCUSSION
Documentation of traditional knowledge

The different ethnic groups of people of Meghalaya and Mizoram states of North East India traditionally prepare and consume soybeans both as fermented and non-fermented food items. Their indigenous knowledge of preparation of fermented soybean products viz. turangbai (Meghalaya) and bekang (Mizoram) were worth documenting as low-cost, high-protein foods and for socio-cultural reasons. These fermented soybean products can be preserved and consumed even after a week or months. Ethnic people might have invented such preservation technique to feed themselves during the scarcity of food (Tamang, 2005a). Information were sought directly from the local elder people and older women of the respective places during survey on ethnic fermented soybean foods, they prepare and consume, their traditional method of preparation, culinary skills and socio-economy of the products, and ethnical value. The native skills of food fermentation have been passed from mothers/fathers to daughters/sons, through the traditional knowledge of the elders, which include grand-mothers/fathers, mothers/fathers, and village elders, self-practice, family tradition, community knowledge and neighbours (Tamang, 2010a).

Microbial isolation, enrichment in appropriate culture media, purification, characterization based on standard taxonomical keys, proper identification, and authentic nomenclature of
microorganisms associated with fermented foods and beverages are important aspects of microbial systematic which ensure the quality control and normalised production of fermented foods (Tamang and Holzapfel, 1999; Tamang, 2012). The isolated, identified and preserved microorganisms from lesser-known ethnic fermented foods and beverages contribute significant database information on unknown microbial gene pool as genetic resources (Tamang and Kailasapathy, 2010). Microorganisms transform the chemical constituents of raw substrates during fermentation into acceptable food products with improved flavour, aroma and texture, enhancing nutritional value and other health benefits (Tamang, 2010b).

In turangbai, the average microbial population of Bacillus spp. was predominant and was present in viable numbers above $10^8$ cfu/g, followed by lactic acid bacteria (LAB) in the range of $10^3$ to $10^4$ cfu/g, load of yeasts ranged upto $10^5$ cfu/g. Total viable counts in turangbai was in the range of $10^8$-$10^9$ cfu/g. In bekang, the average microbial populations of Bacillus spp. was in the range of $10^2$ to $10^8$ cfu/g, the population of LAB was in the range of $10^4$ to $10^5$ cfu/g, load of yeasts was recovered at the level of less than $10^3$ cfu/g. Total viable counts in bekang was $10^8$-$10^9$ cfu/g. Filamentous moulds were not detected in any of the samples analysed. Bacillus subtilis, Enterococcus faecium, Candida parapsilosis and Geotrichum candidum were reported from the
fermentation of kinema (Sarkar et al., 1994). Besides B. subtilis, population of E. faecium and yeasts occurred predominantly in soaked soybeans, which is the starting material for turangbai fermentation. This suggested that LAB and yeasts enter through water sources and wooden grinder (which also revealed the presence of spore formers) thus supplementing the microbes during turangbai fermentation (Sohliya et al., 2009).

Phenotypic identification of Bacillus spp.

Prior to the genotyping, the Bacillus isolates from turangbai and bekang were phenotyped. Initial typing was based upon colony and cell morphology, presence of endospore, Gram staining and catalase test, growth in anaerobic agar and starch hydrolysis. Other characteristics included the fermentation of different carbohydrates and the enzymatic activity of the bacillus isolates using API zym (Biomerieux). Based on the detailed characterisations and identification profiles, three species of Bacillus isolated from turangbai samples were identified as Bacillus subtilis, B. pumilus and B. Licheniformis. Similarly, seven species of Bacillus isolated from bekang samples were identified as B. subtilis, B. pumilus, B. licheniformis, B. sphaericus, B. brevis, B. coagulans and B. Circulans. Like in turangbai and bekang, Bacillus spp. is also responsible for alkaline fermentation in kinema.
(Sarkar et al., 1994; Tamang, 2003), and hawaijar (Jeyaram et al., 2008).

**Genotypic identification of Bacillus spp.**

The traditional methods of phenotyping of Bacillus spp. are labourious and time-consuming to undertake and cannot provide a rapid screening system (Wattiau et al., 2001). These shortcomings of phenotypically based identification methods have led to the development of molecular alternatives based on the microbial genotype or DNA sequence. This approach minimizes problems associated with typability and reproducibility, and importantly, facilitates the assembly of large reference databases (Olive and Bean, 1999). Wang et al. (2003) have stated that comparison of 16S rDNA sequence is one of the most powerful tools for the classification of microorganisms and have provided sequence specific primers as gold standards for the identification of pure cultures of Bacillus spp.

In this study, 16S rDNA region was successfully amplified from genome of all 50 representative strains of Bacillus spp. isolated from turangbai and bekang using the universal 16S rRNA primers: (forward primer 5'AGAGTTTGATCCTGGCTCAG-3', reverse primer 5'AAGGAGGTGATCCAGCCGCA-3' (Weisburg et al., 1991) (Fig 18 a & b). In order to separate similar isolate, all 50 strains of Bacillus were examined by ARDRA. It was previously
reported that ARDRA using universal primers could not separate
*B. licheniformis* from *B. subtilis* (Vaerewijck et al., 2001). Wu et al.
(2006) developed ARDRA (16S rDNA-PCR-RFLP) assay able to
differentiate *B. subtilis* and *B. licheniformis*. The 16S rDNAs were
amplified by PCR using universal primers and the amplified
product was cleaved with the three restriction enzymes, *Hinfl* (Fig
5 a, b), *Rsa I* (Fig 6 a, b) and *Cfo I* (Fig 7 a, b). Each of these
restriction enzymes gave different restriction digestion profiles. The
ARDRA patterns of all of the three restriction enzymes were
combined to achieve different ARDRA groups and 50 strains of
*Bacillus* spp. were grouped into 7 groups (Group I to VII) (Table
7a). However, there are certain limitations to this ARDRA assay,
like the use of universal primers than *Bacillus*-specific primers for
PCR amplification. Thus the subsequent species identification
relied on prior genus identification by phenotypic and biochemical
methods. And also employing many restriction enzymes in the
ARDRA assay is costly in time, labour and expense, though
theoretically the more restriction enzymes used in ARDRA assay,
the more accurate result will be obtained. Heyndrickx also tested
different combinations of restriction enzymes in ARDRA assay and
found that 3 enzymes yielded similar results in terms of species
identification as did 5 enzymes (Heyndrickx et al., 1996).

The ITS-PCR profile clearly differentiated 7 ARDRA groups
into 16 ITS sub-groups (Table 8) (Fig 8 a & b). ITS region was
reported for exhibiting a higher interspecies variation than rRNA genes without intraspecies variation in *Bacillus* (Ouba *et al.*, 2004). Out of 7 ARDRA groups, 4 groups viz. groups I, II, III and V were differentiated into various ITS sub-groups and each ITS sub-groups were characterised by their own distinctive signature bands. ARDRA Groups IV, VI and VII were not differentiated into any sub-groups. The ARDRA and ITS-PCR profiles (banding patterns were scored manually and processed using NTSYSpc software version 2.20f for generation of cluster analysis in a dendrogram based on the Jaccard similarity coefficient [8] and the un-weighted pair group method using arithmetic averages (UPGMA) (Fig. 14). Here, the cluster analysis based on the similarity coefficient revealed that groups I and VII belonged to *B. subtilis* group, group II belonged to *B. licheniformis* group, groups III and IV belonged to *B. cereus* and groups V and VI belongs to *Lysinibacillus fusiformis* group. Groups V and VI were misidentified as *Bacillus sphaericus* during phenotypic identification. *Lysinibacillus fusiformis* is recovered only from the samples of *bekang* and not from *turangbai* samples.

To differentiate at strain level, RAPD-PCR was applied and a huge difference was noticed. Five random primers OPD18 - 5’-GAGAGCCAAC-3’, OPN13 - 5’-AGCGTCACTC-3’, E11 - 5’-CTGGCTTTGTTGATGT-3’, OPD-05 - 5’-TGAGCGGACA-3’, M13 - 5’-GAGGGTGGCGGTTCT-3’ were used. For RAPD-PCR, two major
groups (Group A & B) were formed as a result of grouping based on ITS profiles (banding patterns) (Fig 8 a & b). Group A consisted of 15 strains and group B consisted of 11 strains. RAPD profiles (banding patterns) with random primers OPD18 (Fig. 9), OPN13 (Fig. 10), E11 (Fig. 11), OPD 05 (Fig. 12) and M13 (Fig. 13) were combined and scored manually and processed using NTSYSpc software version 2.20f for generation of cluster analysis in a dendrogram based on the Jaccard similarity coefficient (Sj) and the un-weighted pair group method using arithmetic averages (UPGMA) as shown in Fig 15. The ITS-PCR and RAPD-PCR profiles were also scored manually and processed using NTSYSpc software version 2.20f for generation of cluster analysis in a dendrogram based on the Jaccard similarity coefficient (Sj) and the un-weighted pair group method using arithmetic averages (UPGMA) (Fig 16).

16S rDNA sequence analysis

Based on the result of ARDRA profiles, 16S rDNA amplicons of nine representative strains were selected for sequencing. The sequencing reactions were performed using ABI 3100 DNA sequencer (Applied Biosystems) in both direction with universal primers used for amplification and in case of unsuccessful reactions, internal primers were designed and used by the service providers (GeNei and MWG, Bangalore). The electrophenogram data for 16S rDNA sequence was validated using Chromas 2.33.
software (www.technelysium.com.au). Sequences obtained were matched with previously published bacterial 16S rDNA sequences available in NCBI using BLAST and the Ribosomal Database Project (RDP). Sequence analysis results (Table 7b) revealed that out of nine strains, 1 strain i.e. TS2:B24 (ARDRA group III) representing 6 strains failed in sequencing but showed 100 % similar ARDRA profile with *Bacillus cereus* MTCC 436. Representative *Bacillus* strains TB2: B8a, TB1: B10, BT2: B18 and BAV: B15 matched with *Bacillus subtilis* subsp. *subtilis* (99 % similarity), strain BK1:B13 matched with *B. licheniformis* (99 % similarity) and strain BK1: B18 matched with *B. cereus* (99 % similarity). Strains BAV2:B6 and BAV:B3 matched with *Lysinibacillus fusiformis* (99 % similarity) (Table 7b). Among the species of *Bacillus*, *subtilis* and *cereus* were in highest population in both samples of turangbai and Bekang. Similar result was reported in hawaiyar (Jevaram et al., 2008). Earlier investigation on alkaline fermented foods revealed the presence of considerable level of *B. cereus*, *Staphylococcus aureus* and members of Enterobacteriaceae (Han et al., 2001: Nout et al., 1998).

Hence, the phenotypic identification of bacilli was reconfirmed using molecular identification tools like ARDRA, ITS PCR, RAPD-PCR and 16S rDNA sequencing. The representative isolates after sequencing were confirmed as *Bacillus subtilis* subsp. *subtilis*, *B. licheniformis*, *B. cereus* and *Lysinibacillus fusiformis*. 

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Isolates like TB1:B10 and BK1:B18 which were phenotypically identified as *B. subtilis* were genotypically reconfirmed as *B. cereus*. Similarly, phenotypically identified BAV:B15 (*B. coagulans*) and BAV:B3 (*B. subtilis*) were genotypically reconfirmed as *Lysinibacillus fusiformis*. This discordance in results might have occurred because phenotypic characterisation is sensitivity to culture conditions and bacterial growth phase. Moreover, the rod shape of both *Bacillus* and *Lysinibacillus* might have given rise to similar phenotypic characterization of isolates.

**Identification of LAB**

The population of LAB in *turangbai* and *bekang* were present in viable numbers up to $10^4$ cfu/g and $10^5$ cfu/g, respectively. Classification of LAB into different genera is largely based on morphology, gas production from glucose (Kandler, 1983), mode of glucose fermentation, and growth at different temperatures (Mundt, 1986; Dykes, 1994). Taxonomically diverse species of genus *Enterococcus* and one species of *Lactobacillus* have been identified from the samples of *turangbai*. Based on the detailed characterisations and identification profiles, the strains of LAB isolated from *turangbai* samples were identified as *Lactobacillus brevis*, *Enterococcus faecium*, *E. hirae*, *E. raffinosus*, *E. durans* and *E. cecorum*. Based on the detailed characterisations and identification profiles, the genera and species of LAB isolated from
bekang samples were identified as Enterococcus faecium, E. hirae, E. raffinossus, E. durans and E. cecorum; however, Lb. brevis was recovered from bekang. Enterococcus faecium, which has been isolated from both turangbai and bekang was also reported from kinema (Sarkar et al., 1994; Tamang, 2003). Presence of E. hirae, E. raffinossus, E. durans and E. cecorum have not been reported from the non-salty fermented soybean products so far. Enterococci play beneficial role in production of many fermented foods (Bouton et al., 1998; Cintas et al., 2000). E. faecium appears to pose a low risk for use in foods, because these strains generally harbour fewer recognised virulence determinants than E. faecalis (Franz et al., 2003). The typical enterococci (Enterococcus durans) can be easily distinguished from other Gram-positive, catalase-negative, homofermentative cocci such as streptococci and lactococci by their ability to grow both at 10 and 45 °C, in 6.5 % NaCl, and at pH 9.6 (Franz et al., 2003).

Identification of Yeasts

Though the dominant microflora in all samples of turangbai and bekang was Bacilli and lactic acid bacteria, a sizable number of yeasts were also reported. Saccharomyces cerevisiae, Debaryomyces hansenii and Pichia burtonii were reported from turangbai and bekang. Yeasts strains Candida parapsilosis and Geotrichum candidum were reported from kinema (Sarkar et al.,
1994). Although bacteria (especially *Bacillus* spp.) are considered to have the dominant role in fermentation of soybeans, the contribution of yeasts nevertheless is significant (Romano *et al.*, 2006). *Saccharomyces cerevisiae* was dominant in both the samples of *turangbai* and *bekang*.

**Prevalence of microorganisms**

**Bacilli**

The average prevalence of *bacilli* in *turangbai* collected from different places of Meghalaya showed that *Bacillus subtilis* (55.0 %) was the most dominant followed by *B. licheniformis* (25.5 %), and *B. pumilus* (19.5 %) (Table 17; Fig 19). Prevalence of bacilli in *bekang* was grouped into three main categories viz. Group I: the *licheniformis* group consisted of *B. licheniformis*, *B. coagulans* and *B. circulans*. Group II: the *subtilis* group comprising of *B. subtilis* and *B. brevis* and Group III; the *pumilus* group consisted of *B. pumilus* and *B. sphaericus*. The average prevalence of bacilli in *bekang* collected from different places of Mizoram showed that *subtilis* group (53.8 %) was the most dominant microflora followed *licheniformis* group (32.5 %), and *pumilus* group (13.7 %).

**LAB**

The average prevalence of LAB in *turangbai* showed that *E. faecium* (35.6 %) was the most dominant LAB followed by *E.
durans (20.8 %) then by Lb. brevis (17.4 %), E. cecorum (10.8 %), E. hirae (7.7 %) and E. raffinosus (7.7 %). Mostly Enterococcus faecium was dominant in the samples of turangbai but in some cases Enterococcus faecium showed its dominance (80 % and 60 % in the samples collected from Bara bazaar and Police bazaar respectively) (Fig 20a). In the sample collected from Nongpyuir, the dominant LAB was Lactobacillus brevis (100 %). Enterococcus spp. was the only lactic acid microflora isolated from all the samples of bekang collected from different places. The average prevalence of LAB in bekang showed that E. faecium (38.9 %) was the most dominant LAB followed by E. durans (30.8 %) then by E. hirae (11.7 %), E. raffinosus (9.6 %) and E. cecorum (9.3 %).

Yeast

Yeast was the least dominant microflora found in both turangbai and bekang. The average prevalence of yeast in turangbai showed that Saccharomyces cerevisiae (59.5 %) was the most dominant followed by Debaryomyces hansenii (20.6 %) and Pichia burtonii (19.9 %). Turangbai samples collected from Saldev, Mihmyntdu, Laitjem, Tiuber and Wahiajer showed the absolute dominance of Saccharomyces cerevisiae (100 %) and no other yeasts were recovered (Fig 22b). The average prevalence of yeast in bekang showed that S. cerevisiae (59.8 %) was the most dominant yeast followed by P. burtonii (29.9 %) and D. hansenii (10.3 %).
Samples collected from Zemabawk, Sialsuk, Lamzawl, Kolasib and Bethlem veng showed absolute dominance (100%) of *Saccharomyces cerevisiae*, whereas the samples collected from Kawnpui and Siphir, showed the absolute dominance (100%) of *Pichia burtonii*.

**Occurrence of pathogenic bacteria in turangbai and bekang**

In turangbai, *Bacillus cereus* and enterobacteriaceae were detected but their count was less than $10^2$ cfu/g. These pathogens might have introduced during handling of raw materials for preparation when pH was not low enough to inhibit their growth. Otherwise, no pathogenic bacteria such as *Listeria* sp., *Salmonella* sp., *Shigella* sp. and *Staphylococcus aureus* were detected in any samples analysed. In bekang, counts of *Bacillus cereus* was detected up to $10^3$ cfu/g. Enterobacteriaceae was detected only in few samples of bekang collected from places like Serehip, Ngopa and Kawnpui at the level of $<10^2$ cfu/g (Table 24). *Bacillus cereus* was detected as the only contaminant in the samples collected from Saitual, Armed veng and Aizawl market. Otherwise, no pathogenic bacteria such as *Listeria* sp., *Salmonella* sp., *Shigella* sp. and *Staphylococcus aureus* were detected in any samples analysed. Small number of *Bacillus cereus* in foods is not considered significant (Roberts et al., 1996). Rapid growth of LAB could restrict the growth of other organisms simply by their
physical occupation of available space and uptake of most readily assimilative nutrients (Adams and Nicolaides, 1997). Moreover, lactic acid produced by LAB may reduce pH to a level where pathogenic bacteria may be inhibited or destroyed (Holzapfel et al., 1995; Tsai and Ingham, 1997).

TECHNOLOGICAL PROPERTIES

Technological or technical properties of LAB strains isolated from fermented foods are important criteria for selection of starter cultures to be used in the manufacture of functional foods (Durba-Ozkaya, 2001; Badis, 2004).

Amylolytic and proteolytic activities of species of Bacilli and LAB from turangbai and bekang

In case of turangbai, all the 20 representative strains of Bacillus showed proteolytic activity (showing >2 mm hydrolysis zone in milk agar plate) and their protease activity ranged between 0.7 - 3.2 U/ml (Table 25), while in case of bekang, all the strains of Bacillus except B. subtilis BK1:B18 showed protease activity (0.5–3.0 U/ml) (Table 28). Bacillus subtilis produced the highest proteolytic activity. Garcia et al. (1994) found that proteinases obtained from Bacillus subtilis were highly active and produced bitterness because of their intense proteolytic action on b-casein. Proteolysis induced an increase in free amino acids content (Rasic
et al., 1971), improves the digestibility of proteins (Breslaw and Kleyn, 1973). Most of the bacilli from turangbai showed positive amylolytic activity (showing >2 mm hydrolysis zone in starch agar plate) and their $\alpha$-amylase activity ranged between 0.8 - 5.7 U/ml. But few strains of Bacillus pumilus (TB1:B17, TM1:B5, TP1:B4 and TSA:B15) showed negative starch hydrolysis and $\alpha$-amylase activity (Table 25). Most of the bacilli from bekang showed positive amylolytic activity (showing >2 mm hydrolysis zone in starch agar plate) and their $\alpha$-amylase activity ranged between 0.8 - 4.8 U/ml. But few strains of Bacillus pumilus (TB:B3, BT:B11, BME:B20, BK1:B18, BK1:B15, BK2:B6 and BAV:B12) showed negative starch hydrolysis and $\alpha$-amylase activity (Table 28). Bacillus licheniformis, B. coagulans have been proven to produce thermostable $\alpha$-amylase at alkaline pH (Medda and Chandra, 1980). All the 18 strains of LAB from turangbai, tested for amylolytic activity showed negative $\alpha$-amylase activity but most of them showed negative protease activity with the exception of the few strains of Enterococcus raffinosus (TP2:L11, TS1:L1 and TS1:L8) (showing >2 mm hydrolysis zone in milk agar plate) and their protease activity ranged between 0.9 - 1.1 U/ml (Table 26). All the 20 representative strains of LAB from bekang showed negative $\alpha$-amylase activity. Most of the LAB showed negative protease activity with the exception of the strain Enterococcus raffinosus.
(BT:E3, BK1:L5, BK1:L7 and BK1:L9) which showed positive protease activity ranged between 0.8 – 1.3 U/ml (Table 27).

**Enzymatic profiles**

Enzymatic profiles of *Bacillus* strains were assayed using the API-zym (bioMérieux, France) galleries (Tables 29). Each of the predominant *Bacillus* strain produced a wide spectrum of enzymes. The use of the API-zym technique has been reported (Arora et al., 1990) as a rapid and simple means of evaluating and localising 10 different hydrolases of microorganisms associated with dairy fermentations. This method is also of relevance for selection of strains as potential starter cultures based on superior enzyme profiles especially peptidases and esterases, for accelerated maturation and flavour development of fermented products (Tamang et al., 2000; Kostinek et al., 2005). Absence of proteinases (trypsin and chymotrypsin) and presence of strong peptidase (leucine-, valine-, and cystine-arylamidase) and esterase-lipase (C4 and C8) activities produced by the predominant *Bacillus* strains isolated from fermented soybean products of Meghalaya (*turangbai*) and Mizoram (*bekang*) are possible traits of desirable quality for their use in production of typical flavour. All the fifteen strains tested showed relatively moderate esterase (C4) and phosphohydrolase activity. However, they showed no detectable proteinase activity with the methods.
applied. Alkaline phosphatase activity was exhibited moderately by all the strains of Bacillus tested. Alkaline phosphatase dephosphorylates inactive derivatives of many antibiotics in the final step of biosynthesis and a direct relationship between intracellular enzyme level and antibiotics formation were well established (Majumdar and Majumdar, 1971). Lipase (C14) activity was detected only in few strains like B. subtilis BD1:B1 (bekang), B. subtilis TB2:B13 (turangbai), B. licheniformis BME:B16 (bekang), B. licheniformis TP1:B5 (turangbai).

**PGA-production by Bacillus species of turangbai and bekang**

Poly-glutamic acid (PGA) is one of the few naturally occurring polypeptides, which are not synthesized by ribosomal proteins (Oppermann-Sanio and Steinbüchel, 2002). The polymer is produced by several Bacillus sp. as an extracellular viscous polymer (Kunioka and Goto, 1994; Ko and Gross, 1998). It is safe for eating as a viscosity element of fermented soybean products such as **changkookjang** and **natto** (PGA is completely biodegradable and water-soluble and non-toxic to human (Yoon et al., 2000). Screening of the production of poly-glutamic acid (PGA) by selected strains of Bacillus isolated from turangbai and bekang was carried out and tabulated in Table 30 and 31, respectively. Bacillus subtilis (natto) Miura strain was taken as a reference strain, which produced 3.6 mg/ml of PGA in the PGA.
Medium (Kunioka and Goto, 1994). On comparison to the PGA produced by the reference strain (3.6 mg/ml), *B. subtilis* TS1:B25 (*turangbai*) and *B. subtilis* BT:B9 (*bekang*) accounted for the highest production of PGA (2.8 mg/ml each) amongst the other strains tested (Table 30 and 31) which suggests that *B. subtilis* is the most potent PGA producer than the other *Bacillus* sp. *B. subtilis* (*natto*) produces a unique capsular polymer of glutamic acid with γ-peptide linkage, poly-γ-glutamic acid (PGA). The most striking feature of the γPGA produced by *B. subtilis* (*natto*) includes its very large molecular mass of over 10^8 Da and the presence of both L- and D-glutamic acids (Leonard et al., 1958, Saito, et al., 1974). *Bacillus subtilis* and *Bacillus licheniformis* are the most widely used industrial producers of γ-PGA. The γ-PGA product is secreted into the medium and may protect the organism from the harsh environmental conditions or serve as a carbon, nitrogen, energy source or biofilm formation enhancer (Stanley and Lazazzera, 2005).

**Degradation of poly-glutamic acid (PGA) by LAB strains**

Thirty strains of randomly selected LAB isolated from *turangbai* were tested for their ability to degrade poly-glutamic acid (PGA) but none of the strains were able to degrade it (Table 32). Similarly, thirty strains of selected cocci lactics isolated from *bekang* were tested for their ability to degrade PGA, and found that
none of the strains degraded PGA. Moreover, PGA degradation by lactic acid bacteria has not been reported so far. Whereas, Chunhachart et al. (2006) have reported that the enzyme γ-Glutamyl hydrolase purified from culture broth of *Bacillus* sp. isolated from Thai *thua-nao*, degraded γ-polyglutamic acid (PGA) to a hydrolyzed product of only about 20 kDa (with D- and L-glutamic acid in a ratio of 70:30).

**Acidification and coagulation activities**

Acidification is an important technological property in relevance of selection for starter culture among the LAB (de Vuyst, 2000). Effect of acidification and coagulation by the LAB strains isolated from *turangbai* (Table 34) and *bekang* (Table 35) were tested. *Enterococcus faecium* TB1:L5 and TSB:L2 (*turangbai*) showed the lowest acidification value of pH 4.3, followed by *E. durans,* *E. faecium* and *E. cecorum* dropping the pH upto 4.4 (Table 34). In case of *bekang,* *E. cecorum* BAV2:E7 and *E. faecium* BME:L4 showed the lowest acidification value of pH 4.3, followed by *E. faecium,* *E. cecorum* and *E. hirae* lowering the pH upto 4.4 (Table 35). About 45 % of LAB strains of *turangbai* and about 49 % of LAB strains of *bekang* caused coagulation of milk at 30 °C with a significant drop in pH. All strains of *Enterococcus faecium* and *E. cecorum* coagulated skim milk (Table 34 and 35). Coagulation of milk by LAB strains shows their potential as
starters or adjunct cultures in the production of fermented products. The casein degradation initiated with milk clotting peptidases and proteinases, which produce peptides and amino acids (Mäyra-Mäkinen and Bigret, 1998).

Antimicrobial activities

Most of the LAB strains showed antimicrobial activities against a number of potentially pathogenic Gram-negative and Gram-positive bacteria (indicator strains), showing antagonisms. 23 strains out of 58 strains of LAB (turangbai) and 37 strains out of total 60 strains of LAB (bekang) tested for antagonism, showed the clear inhibition zones measurements by scale of more than 4 mm in agar spot plates in the applied method (table 36 and 38). This reveals that antimicrobial properties of functional LAB can reduce the number of other undesired microorganisms in soybean products and simultaneously perform an essential role in the preservation of a food product for human consumption, by fermentation. However, the cell-free supernatant fluid extracts of LAB strains isolated from turangbai and bekang could not produce bacteriocin (table 37 and 39) under the applied condition. Lactic acid bacteria compete with other microorganisms by screening antagonistic compounds and modifying the micro-environment by their metabolism (Lindgren and Dobrogosz, 1990; Tagg, 1992). Production of bacteriocin depends on a number of intrinsic and
extrinsic factors including redox potential, water activity, pH and temperature (Yang and Ray, 1994; Delgado et al., 2005). A number of Gram-positive pathogenic bacteria including *Staphylococcus aureus* have been found sensitive to bacteriocin of *Lactobacillus* (Ticháček et al., 1992; Sudirman et al., 1993; Niku-Paavola et al., 1999).

**Screening of biogenic amines-producing LAB**

Strains of LAB isolated from *turangbai* and *bekang* were not able to produce biogenic amines in the biogenic screening medium containing precursor amino acids (tyrosine, lysine, histidine and ornithine) in the method applied. Biogenic amines are the organic bases with aliphatic, aromatic or heterocyclic structures that can be found in several foods, in which they are mainly produced by microbial decarboxylation of amino acids, with the exception of physiological polyamines (Silla Santos, 1996). In foods, biogenic amines are mainly generated by decarboxylation of the corresponding amino acids through substrate specific enzymes of the microorganisms present in foods (ten Brink et al., 1990; Straub et al., 1995). The inability of most strains of LAB to produce biogenic amines in tested fermented soybean products of North East India is a good indication of their acceptability and their potential for the possible development as starter culture. The production of biogenic amines by LAB to be selected as starter
cultures is not a desirable property (Buchenhûskes, 1993; Holzapfel, 1997). Histaminine, precursor of biogenic amine has been recognised as the causative agent of scombroid poisoning (histamine intoxication), whereas tyramine has been related to food induced migraines and hypertensive (Taylor, 1986; Bover-cid and Holzapfel, 1999). Samples with moderate, high or very high levels of biogenic amines could be considered as products of less quality and their consumption could be unhealthy for sensitive individuals (Latorre-Moratalla et al., 2007).

**Hydrophobicity of the LAB strains**

Bacterial adherence to hydrocarbons such as hexadecane, proved to be a simple and rapid method to determine cell surface hydrophobicity (Rosenberg et al., 1980; van Loosdrecht et al., 1987; Ding and Lammier, 1992; Vinderola et al., 2004). A few strains of LAB isolated from nurangbai and bekang, had more than 70 % hydrophobicity (Table 42 and 43), indicating their hydrophilic nature.

Percent of hydrophobicity greater than 70 % was arbitrarily classified as hydrophobic (Nostro et al., 2004). The high degree of hydrophobicity of the LAB strains, isolated from fermented soybean products, probably indicates the potential of adhesion to gut epithelial cells of human intestine, advocating their 'probiotic' character (Holzapfel et al., 1998), provided these strains are
consumed in a viable state. The ability to adhere to the intestinal mucosa is considered one of the main criteria in the selection of potential probiotic culture (Apostolou et al., 2001; Shah, 2001; Holzapfel and Schilliinger, 2002). Functional effects of probiotic bacteria include adherence to the intestinal cell wall for colonization in the gastrointestinal tract (GIT) with capacity to prevent pathogenic adherence or pathogen activation (Bernet et al., 1993; Salminen et al., 1996). The behaviour of LAB could be dependent on interfacial processes and thus on cell surfaces, physicochemical properties and chemical composition (Gatti et al., 1997, Boomaert and Rouxhet, 2000, Gómez-Zavaglia et al., 2002). E. faecium TM2:Lo (turanghai) and E. faecium BAV:E2 (beikang) showed the highest degree of hydrophobicity of 72.7% and 71.6% respectively (table 42 and 43).

Degradation of antinutritive factors

All plants have some anti-nutrient properties, but soybeans unlike other legumes are rich in anti-nutrients like phytic acid/phytates and oligosaccharides like raffinose and stachyose. Phytic acid has the strong ability to chelate multivalent metal ions, especially zinc, calcium, and iron. The binding can result in very insoluble salts that are poorly absorbed from the gastrointestinal tract, which results in poor bioavailability (BV) of minerals (Zhou and Erdman Junior., 1995). It has a strong affinity for zinc, a
mineral that supports wound healing, brain development, protein synthesis, immunity etc. Antinutritive factors such as phytic acids and oligosaccharides are of particular significance in unbalanced cereal-based diets (Holzapfel, 2002; Fredrikson et al., 2002). Oligosaccharides such as raffinose, stachyose and verbascose cause flatulence, diarrhea and indigestion (Abdel Gawad, 1993; Holzapfel, 1997). Due to these nutritional consequences, the degradation of antinutritive factors in food products by fermentation is desirable as reported for a number of foods of plant origin (Chavan and Kadam, 1989; Mbogua et al., 1992). In case of turangbai, 34.9% of LAB strains degraded phytic acids, 43% degraded raffinose and 19% degraded both phytic acid and raffinose but none of the strains degraded stachyose in the applied method (Table 44). Whereas, in case of bekang, 45% of LAB strains degraded phytic acid, 35% degraded raffinose and 20% degraded both phytic acid and raffinose but none of the strains degraded stachyose in the applied method (Table 45). This proves that the fermented soybean products like turangbai and bekang contains lowest level of phytic acid and oligosaccharides that make them fit for consumption.

Antioxidant capacity and total phenol content

Phenolic compounds are considered as the most important antioxidative components of herbs and other plant materials, and
good correlation between the concentration of plant phenolics and the total antioxidant capacity has been reported (Pellegrini et al., 2000). Typical phenolics that possess antioxidant activity are known to be mainly phenolic acids and flavonoids. In cellular defense system, scavenging of free radicals is an important issue affiliated by utilization of both exogenous and endogenous antioxidants because the increase in production of free radicals has been reported to cause damage to cell membranes, enzymes, DNA, lipids, and proteins, impairing their function (Gu et al., 1998). Although the body possesses defense mechanisms as enzymes and antioxidant nutrients (Halliwell et al., 1995), continuous exposure to chemicals and contaminants may lead to an increase in the amount of free radicals in the body beyond its capacity to control them and cause irreversible oxidative damage (Tseng et al., 1997). The total phenolic content (TPC) values of *kurungbai* samples collected from different places ranged from 2.1 ± 0.1 to 3.7 ± 0.1 mg GAE/g fresh weight and the highest TPC value was shown by the sample collected from Shyrman (3.7 ± 0.1 mg GAE/g fresh weight) (table 46). Similarly, in case of *bekang*, TPC values ranged from 2.6 ± 0.1 to 4.2 ± 0.3 mg GAE/g fresh weight and the highest TPC value was shown by the sample collected from Ngopa (4.2 ± 0.3 mg GAE/g fresh weight) (table 47). Pourmorad et al. (2006) reported that the extract which contained highest amount of flavonoid and phenolic compounds, exhibited
the greatest antioxidant activity. The polyphenolic compounds, like flavonoids and phenolic acids, commonly found in plants (Brown and Rice-Evans, 1998) and their epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent (Halliwell, 1994).

A lower value of antioxidant activity as compared to the standard (Ascorbic acid, IC₅₀ = 2.69 ± 0.0 µg/ml) in scavenging of stable free radical DPPH (2,2-diphenyl-1-picrylhydrazyl), was found in samples of turangbai and bekang. The highest DPPH scavenging activity of turangbai was shown by the sample collected from Shyrmang (IC₅₀ = 515.7 ± 35.1 µg/ml) and in case of bekang, it was shown by the sample collected from Ngopa (IC₅₀ = 456.7 ± 30.6 µg/ml) which significantly correlated with their TPC values (table 46 and 47). ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) radical scavenging activity of the samples of turangbai and bekang were found to be lower than that of the standard (Ascorbic acid, IC₅₀ = 11.25 ± 0.4 µg/ml) used. The highest ABTS scavenging activity of turangbai was shown by the sample collected from Shyrmang (153.3 ± 25.2 µg/ml) whereas, in case of bekang, the highest value was recorded in sample collected from Serchhip (456.7 ± 30.6 µg/ml) (table 46 and 47). Wang et al. (2007) reported that DPPH and ABTS radical scavenging activity of
douchi (a Chinese traditional salt-fermented soybean food) extracts increased significantly during the pre-fermentation ($p < 0.05$) but the activity decreased during the douchi fermentation due to high salt addition. Moktan et al., (2008) reported that the TPC of kinema fermented with Bacillus subtilis was 144% higher than that of cooked non-fermented (CNF) soybean (3.3 mg/g dry weight) and it was found to be a better free radical scavenger, which increased in a time and dose dependent manner than the CNF soybean. The antioxidative activity and chemical components of the traditional fermented soybean products like “miso”, “natto” and tempeh incubated with Aspergillus oryzae, Bacillus natto and Rhizopus oligosporous respectively, have proved to be more stable against lipid peroxidation than steamed soybeans. This result indicates that antioxidative compounds can be produced by fermentation (Esaki et al., 1993). Methanolic extracts of tua-nao, a Thai fermented soybean product, exhibited antioxidant and free radical scavenging properties and it was also found that there was a strong relationship between total phenolics content and antioxidant activity (Dajanta et al., 2011). Chungkookjang (a traditional Korean fermented soyfood) made by fermenting large black soybean (LBS) at 42 °C for 72h exhibited higher total phenol and isoflavone contents and thus high antioxidant and free radical scavenging activity than the small black soybean (Shon et al., 2007). Wang et al., (1998) showed that some compounds which
have ABTS (2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)) scavenging activity may not show DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity. All these reports firmly support the antioxidant potentials of the extracts of turangbai and bekang, observed in the present study is due to the presence of phenolic compounds.

Turangbai and bekang preparation by selected starter culture(s)

Use of standard starter culture is not a practice in the North Eastern region of India, especially in Meghalaya and Mizoram except in alcoholic beverage production (Thapa and Tamang, 2004). The rationale behind is to use starter culture in order to supplement the natural microflora of turangbai and bekang. Sensory evaluations were carried out in order to choose the best culture or their combination. It was found that turangbai produced using starter 'D'- a mixture of pure culture strains of B. subtilis TS2:B24, B. licheniformis TSB:B13 and B. pumilus TSA:B15 and bekang produced using starter 'H'- a mixture of pure culture strains of B. subtilis BT:B9, B. licheniformis BK1:B13 and B. pumilus BK2:B6 selected on the superior technological property as mentioned in the result section, at 40 °C for 48 h, organoleptically scored the highest acceptability among the judges. None of the strains of Bacillus used singly as starters could produce organoleptically acceptable turangbai and bekang. The
sensory evaluation result showed that *turangbai* and *bekang* prepared in the laboratory by cell suspension mixture of *Bacillus* spp. was more acceptable than conventionally prepared products.

Application of starter cultures may appear appropriate in *turangbai* and *bekang* production at household level since it is cost-effective and may contribute to effective control and safeguarding of the fermentation process. *Turangbai* and *bekang* prepared by using a starter culture had thus advantages over the traditional method, which resulted in a shorter fermentation time that eliminates the chance of growth of contaminants. Hygienic conditions, maintaining consistency with better quality and flavour. The final product is not always consistent in natural fermentation, the use of a mixed starter culture could provide more consistent fermentations and products of higher quality (Gardner et al., 2001; Zorba et al., 2003). Though, optimised process condition is always superior and advantageous than the conventional method, however, introduction and replacement of natural and easily operated traditional technology may be difficult to change for the producers or rural populace (Holzapfel, 1997). Authentic identity of functional microbes in fermented foods is necessary to develop the starter cultures isolated from conventionally prepared foods (Geisen and Holzapfel, 1996; Tamang and Holzapfel, 1999). Preservation and safeguarding of foods are still major objectives of fermentation (Holzapfel, 2002).
Yet, other aspects such as wholesomeness, acceptability and overall quality have become increasingly important and valued features to account of the substrate, technical properties of the strain, food safety requirements and quality expectations (Holzapfel et al., 2003).

Conclusion

Turangbai and bekang are ethnic fermented soybean foods of Meghalaya and Mizoram respectively, and are traditionally prepared by women. Diversity of ethnic fermented foods of North-Eastern states of India is related to diversity of ethnicity with unparallel food culture of each community. The traditional knowledge of ethnic people for turangbai and bekang preparation was worth documenting. Scientific knowledge on these products is unknown outside the North East region of India. Microorganisms associated with these two products were bacilli \textit{Bacillus subtilis}, \textit{B. licheniformis}, \textit{B. cereus} and \textit{Lysinibacillus fusiformis}. Lactic acid bacteria (LAB) \textit{Lactobacillus brevis} (only in turangbai), \textit{E. faecium}, \textit{E. hirae}, \textit{E. raffinossus}, \textit{E. durans} and \textit{E. cecorum} and yeasts: \textit{Saccharomyces cerevisiae}, \textit{Debaryomyces hansenii} and \textit{Pichia burtonii}. The predominant microorganism in both the product is bacilli followed by LAB and yeast.

This study revealed that strains of bacilli along with LAB and yeast play important and partly complex role in this
traditional fermentation process by virtue of their technological or functional properties related to a specific and partly a wide enzyme spectrum, their acidifying capacity and antimicrobial activities of LAB (though bacteriocin production was not observed), degradation of antinutritive factors, probiotic properties (adherence potential indicated by a high degree of hydrophobicity), non-producers of biogenic amines, poly-glutamic acid (PGA) production by bacilli, enhancement of antioxidant activity, bio-enrichment of nutritional value, bio-availability of minerals, etc. Due to possession of superior functional properties, some of the strains of bacilli can be used as starter culture(s) for controlled and optimized production of fermented soybean products typical of the Meghalaya and Mizoram. Turangbai and bekang prepared by a mixed pure culture strains of bacilli were more acceptable and had many advantages over the conventionally prepared products. Native microorganisms with vast biological importance and potential genetic resources are associated with ethnic fermented foods, which should be preserved before they are forced to disappear.