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International Journal of FERMENTED FOODS

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ABOUT THE JOURNAL

International Journal of Fermented Food aims to publish original articles which contain results of research in food science related to fermentation as a method of food processing and preservation. Fermentation is a process of food preservation with great potential. It is an environment friendly process, consumes less energy and produces less waste. It is easy to manage both under simple household conditions and in an industrial scale. It could be applied to a wide variety of raw materials to produce a variety of different finished food products. It has the advantage of being generally regarded as safe (GRAS) and offers immense opportunities for production of novel products which can be classified as “organic foods”, “natural foods”, “health foods”, “convenience foods”, “ethnic foods”, “neutraceuticals”, “functional foods” and not to forget “foods for clinical nutrition”. Cereals, pulses, root crops, vegetables, fruits, meat and fish are preserved by one or other method of fermentation in some part of the world.

The International Journal of Fermented Foods (IJFF) will publish high-quality research articles that meet the general criteria of significance and academic excellence, written in English, in all areas of the subject Fermented Foods. The journal will be published half yearly to start with and then converted to quarterly in two years. It will also accommodate review articles on any area of fermented foods viz. technology, microbiology, biochemistry, nutrition, health marketing, etc. It will also publish work on probiotics, prebiotics, synbiotics, neutraceuticals and related clinical studies.

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Editorial

Publication of a good quality journal is not an easy task. Any new journal has to undergo a vicious cycle of impact factor and quality articles. When you start a new journal, obviously you do not have any impact factor. In absence of impact factor, it is very difficult to get good quality articles. In this process, generally the new journal receives second or third quality articles and when the publisher put it for impact factor, it receives very low impact factor!

We are happy to put this third volume of our International Journal of Fermented Foods in your hands. Our publisher will now process the papers for assessing the journal for the impact factor. We had teething problems in convincing the scientists to send us good papers so that we get better impact factor too. We need to wait and watch now that what we get. The editorial board is highly thankful to all those researchers who contributed their best work in the first three volumes. Dr. Pratima Khandelwal, Executive Editor, did untiring job of collecting articles, sending for peer review, communicating with publishers and finally checking the proof. The entire editorial board appreciates her efforts in the tough time of journal.

Among the fermented foods, activities of probiotics are very much in lime light. All over the world, several laboratories are engaged in working with probiotics and hence we also received most of the papers on probiotics. Even after EFSA's strict policy on health claims, the work on probiotics had not gone down. The regulations in many countries, including India are not clear and we hope that in near future, this grey area will see some clarity. Our journal is not limited to probiotics and hence we request the scholars to send us articles on all aspects of fermented foods. We should dig out information about lesser known fermented foods from remote areas; we can pen down traditional processes and also talk about how they play an important role in day to day life of the society. Let us try to act on theme line of SASNET- Fermented Foods "A network for connecting fermented foods to health status and social well-being"

Jashbhai B. Prajapati
Associate Editor &
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Research Paper

Functional Properties of *Tungrymbai* and *Bekang*, naturally fermented soybean foods of North East India

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Abstract

We determined some functional properties of species of *Bacillus* and lactic acid bacteria (LAB) previously isolated from *tungrymbai* and *bekang,* naturally fermented soybean foods of India which included enzymatic activities, production and degradation of poly-γ-glutamic acid (PGA), acidifying capacity, degradation of antinutritive factors, antimicrobial activities, ability to produce biogenic amines, degree of hydrophobicitys, antioxidant activity and total phenol contents of the products. *Bacillus* spp. showed wide spectrum of enzymatic activities. *Bacillus subtilis* TS1:B25 (*tungrymbai*) and *B. subtilis* BT:B9 (*bekang*) accounted for the highest production of PGA (2.8 mg/ml each) amongst the other strains tested. Though LAB showed antimicrobial activities, none of them produced bacteriocin and biogenic amines under the applied condition. *Enterococcus faecium* TM2:L6 (*tungrymbai*) and BAV:E2 (*bekang*) showed the highest degree of hydrophobicity of 72.7% and 71.6%, respectively. LAB strains were able to degrade phytic acid and oligosaccharides, showing their ability to degrade anti-nutritive factors. *Tungrymbai* and *bekang* possess antioxidant and free radical (DPPH and ABTS) scavenging activity. This is the first report on functional properties of *tungrymbai* and *bekang*.

Practical Applications: This study was to determine some functional properties of microorganisms previously isolated from *tungrymbai* and *bekang,* naturally fermented soybean foods of India. *Bacillus* spp. showed wide spectrum of enzymatic activities. *Bacillus subtilis* strains accounted for the highest production of PGA. *Enterococcus faecium* TM2:L6 (*tungrymbai*) and BAV:E2 (*bekang*) showed the highest degree of hydrophobicity of 72.7% and 71.6%, respectively. *Tungrymbai* and *bekang* possess antioxidant and free radical (DPPH and ABTS) scavenging activity.

Keywords: Functional properties, fermented soybeans, *tungrymbai*, *bekang,*

Soybean is a major leguminous crop in the world, and its utilization as foods are mostly confined to Asia. Soybean was probably introduced to India from China through the Himalayas several centuries ago and some believe that soybeans were also brought via Myanmar by traders from Indonesia (Shurtleff and Aoyagi, 2010). Preparation and consumption of sticky, non-salty, flavorsome fermented soybean foods are the traditional wisdom of the people of South-East Asia and they remain a distinct food culture of the people (Tamang, 2010). *Tungrymbai* and *bekang* are naturally fermented ethnic soybean foods of Meghalaya and Mizoram states in India, respectively (Tamang *et al*., 2009a). On the basis of a combination of phenotypic and genotypic characterization, strains of bacilli isolated from *tungrymbai* and *bekang* were identified as *Bacillus subtilis, B. pumilus, B. licheniformis, B. cereus, B. coagulans, B. circulans, B. brevis* and *Lysinibacillus fusiformis* (Chettri, 2012). Similarly, based on phenotypic characterization profiles, strains of lactic acid bacteria (LAB) were identified as *Lactobacillus brevis* (only in *tungrymbai* samples), *Enterococcus faecium, E. durans, E. hirae, E. raffinossus* and *E. cecorum* (Chettri, 2012). Functional or technological properties of LAB isolated from fermented foods are important criteria for selection of starter cultures to be used in the manufacture of functional foods (Durlu-Ozkaya, 2001; Badis, 2004). The present paper is aimed to study the functional properties of *Bacillus* species and LAB previously isolated from *tungrymbai* and *bekang* such as enzymatic activities, production and degradation of poly-γ-glutamic acid (PGA), acidifying capacity, degradation of antinutritive factors, antimicrobial activities, ability to produce biogenic amines, and to determine the degree of hydrophobicity. Antioxidant activity (DPPH and ABTS) and total phenol contents of the products were also determined.

Materials and Methods

Collection of samples

Samples of *tungrymbai* (21) were collected from various places of Meghalaya - Bara bazaar, Police Bazar, Sohra, Mylliemgat, Nongpyuir, Laitjem, Saldew, Jowai, Mihmyntdu, Shyrmang, Tiuber, Wahiajer and Dawki. Samples of *bekang* (22) were collected from different villages and market places of Mizoram - Thakthing bazaar, Melthum, Saitual, Bethlem Veng, Armed veng, Zemabawk, Aizawl market, Selesih, Siphir, Sialsuk, Serchhip, Kolasib, Kawnpuii, Ngopa and Lamjawl. Samples were collected aseptically in pre-sterile poly-bags and were, sealed and stored at –20° C for further analyses.

Reference Strains

References strains used as indicator strains for antimicrobial activity were: *Bacillus cereus* CCM 2010 obtained from Czechoslovak Collection of Microorganisms,

Brno, Czechoslovakia (CCM), *Enterobacter agglomerans* BFE 154, *Klebsiella pneumoniae* subsp. *pneumoniae* BFE 147 and *Pseudomonas aeruginosa* BFE 162 obtained from Institute of Hygiene and Toxicology, Karlsruhe, Germany (BEF), *Listeria innocua* DSM 20649 and *Listeria monocytogenes* DSM 20600 obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany (DSM), and *Staphylococcus aureus* S1 obtained from Food Microbiology Laboratory, Sikkim Government College, Sikkim University, Gangtok, India (FMR). Commercial starter *B. subtilis* (*natto*) Miura strain for production of *natto*, fermented soybean food of Japan, was obtained from Tokyo, Japan. All reference strains were propagated in nutrient agar (M002, HiMedia, India) and were maintained as frozen stocks at –20°C in 15 % glycerol.

Bacillus and LAB strains from tungrymbai and bekang

Previously isolated and identified strains of *Bacillus* and LAB from *tungrymbai* and *bekang* were used for determination of some functional properties. These cultures included: Bacilli- *Bacillus subtilis, B. pumilus, B. licheniformis, B. cereus, B. coagulans, B. circulans, B. brevis* and *Lysinibacillus fusiformis,* and LAB- *Enterococcus faecium, E. durans, E. hirae, E. raffinossus* and *E. cecorum* (Chettri, 2012).

Amylolytic activity

Surface-dried plates of starch agar (Gordon *et al.,* 1973) were streaked with 24 h-old cultures, incubated at 30° C for 4 days (LAB) and 37° C for 2 days (bacilli). After incubation the plates were flooded with iodine solution for 15-30 min and examined the clear zone underneath (after the growth was scrapped off) for amylolytic activity.

Proteolytic activity

Surface-dried plates of milk agar (Gordon *et al.,* 1973) were streaked with 24 h-old cultures, incubated at 30°C for 4 days (LAB) and 37°C for 2 days (bacilli), and examined for any clearing of casein around and underneath the growth for assessment of proteolytic activity.

α-Amylase activity assay

The blue value method of Fuwa (1954) as modified by Kawaguchi *et al.,* (1992) was followed for determination of α-amylase activity. Cultures were grown on broth medium (1.0% soluble starch, 1.0% beef extract, 1.0% peptone, and 0.3% NaCl, pH 7.0) on a rotary shaking incubator at 30° C at 180 rev/min for 48 h. The cultures were immediately centrifuged at 17,000 rpm for 10 min. The enzyme solution was diluted to an appropriate concentration. The enzyme solution and

1.5% soluble starch dissolved in 0.1M Tris-HCl buffer (pH 7.0) were pre-incubated separately at 37°C for 5 min in water-bath incubator. Then, the reaction mixture was started by adding 1 ml of 1.5% soluble starch (HiMedia RM089) to 0.5 ml enzyme solution and incubated at 37°C for 10 min. The reaction was stopped by the addition of 2.5 ml of stop solution (0.5 N acetic acid-0.5 N HCl 5:1). The 100 ml of the reaction mixture was added to 5 ml of 0.01% I_2 – 0.1% KI solution, left at room temperature for 20 min and the absorbance of the resulting solution was measured at 660 nm in UV-VIS Spectrophotometer (Analytik Jena, Germany). One unit of α-amylase activity (dextrinizing power) was defined as the amount of α-amylase which produced 10% reduction in the intensity of blue colour at the above conditions.

Protease activity assay

Protease activity was measured by a modification of the method of Maeda and others (1993). Cultures were grown in phytone broth (Nagai *et al.,* 1994) on a rotary shaking incubator at 30°C at 180 rev/min for 72 h. Cultures were immediately centrifuged at 17,000 rpm for 10 min. The enzyme solution was diluted to an appropriate concentration. Then, the enzyme solution and the substrate solution containing 1% Azocasein (Sigma Chemical Co., USA) was dissolved in 0.1 M phosphate buffer, (pH 6.8) were pre-incubated separately at 37°C for 5 min in a water-bath incubator (Remi, India). The enzyme reaction was started by adding 2 ml of 1% Azocasein to 1 ml of enzyme solution and incubated at 37°C for 20 min. The reaction was quenched by the addition of 2.5 ml of 10% (w/v) trichloroacetic acid. After centrifugation at 15,000 rpm for 10 min, 2 ml of supernatant was neutralized with equal amount of 1N NaOH and the absorbance was measured at 450 nm in UV-VIS Spectrophotometer (Analytik Jena, Germany). One unit of protease activity was defined as the quantity required increasing the absorbance by 0.1 under the above conditions.

Enzymatic profile by API-zym

The enzymatic profile of LAB and yeast isolates were assayed following the method of Arora and others (1990) using API-zym (bioMérieux, France) galleries by testing for the activity of the following 19 enzymes: alkaline phosphatase, esterase (C4), esterase lipase (C8), lipase (C14), leucine-, valine- and cystine-arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. LAB cultures were grown on MRS broth, and yeast cultures were grown on YM broth, respectively and cells were harvested in 2 ml sterile distilled water which was used to prepare suspension of 10^7 cells/ml. The strip was unpacked and 2 drops of cell suspensions were inoculated in each cupule of the strip containing ready-made

enzyme substrates and incubated at 30ºC for 6 h. After incubation, 1 drop of ready-made zym-A and zym-B reagents was added and observed for colour development based on the manufacturer's colour chart.

Screening of poly-γ-glutamic acid (PGA) production

Bacterial production of poly-γ-glutamic acid was screened following the method described by Nagai *et al.,* (1997) and Meerak *et al,.* (2007), with modification. Strains of *Bacillus* were grown at 37ºC for 18-24h with shaking on a rotary shaking incubator at 175 rp/min in a 200 ml conical flask containing 100 ml of PGA medium which consisted of 2.0% glucose, 2.0% sodium glutamate, 1.0% $(NH_4)_2SO_4$, 0.1% Na_2HPO_4, 0.1% KH_2PO_4, 0.05% $MgSO_4.7H_2O$, 0.002% $Mn(Cl_2)_4{\cdot}H_2O$, 0.005% $FeCl_3{\cdot}7H_2O$ (Kunioka and Goto, 1994), and 0.5 mg/ml of biotin (pH 7.5). The culture was centrifuged at 15,000 rpm at 4ºC for 30 min to obtain the supernatant, in which PGA is present. To one volume of supernatant two volume of ethanol was added to precipitate PGA. The fibrous precipitated PGA was collected with glass rod and washed with 10 ml of ethanol. Ethanol was then evaporated to obtain PGA.

Degradation of poly-γ-glutamic acid (PGA)

Screening of LAB for degradation of poly-glutamic acid was performed following the method described by Tanaka *et al.* (1993). Strains were grown in MRS broth (M369 HiMedia, India), for 18-24h at 30ºC. The isolates were streaked or spread on MRS agar plates containing 0.5% PGA solution (pH 4.5), and incubated at 30ºC for 2-3 days. The plates were flooded with 5 ml of 18 N H_2SO_4 and allowed to stand for 30 min at room temperature. The presence of halo around the colony determines the degradation of PGA.

Acidification and coagulation

Effect of acidification and coagulation of LAB strains were assayed by inoculating 10% skim milk with 24 h old cultures (RM1254, HiMedia) (centrifuged at 8,000 *g* for 20 min and sterialized at 110°C for 10 min) at 1% level, and incubated at 30° C. Observation was made for commencement of clotting, and pH was measured after 72 h of incubation (Olasupo *et al.,* 2001).

Phytic acid degradation

Ability of LAB isolates to degrade phytic acid was determined on a synthetic phytic acid screening medium (Holzapfel, 1997), containing calcium phytate (Sigma, USA) as sole phosphate source. Control was prepared without calcium phytate. In preparing the medium, phytate and salts are added separately. After adding glucose, Na-citrate, magnesium sulfate, manganese sulfate and ferrous

sulfate to the phytate solution, the pH was adjusted to 6.0 and the medium was autoclaved. Vitamins, amino acids and nucleotides were filter sterialised and added to the medium before plating. The pH of the medium was finally adjusted between 5.8-6.0. The plates were streaked with 24 h-old broth culture and incubated aerobically at 30°C for 5 days. Clear zone around the colony of the test organism indicated a positive reaction.

Degradation of oligosaccharides

Screening of LAB for degradation of oligosaccharides such as stachyose and raffinose were performed in MRS broth without beef extract (pH 6.4) containing 2% stachyose and 1% raffinose (instead of glucose), respectively, and 0.004% chlorophenol red as indicator. Inoculation was followed by incubation at 30°C for 3 days (Holzapfel, 1997).

Antimicrobial activity

The LAB isolates were screened for antimicrobial activity against *Bacillus cereus* CCM 2010, *Enterobacter agglomerans* BFE 154, *Klebsiella pneumoniae* subsp. *pneumoniae* BFE 147, *Listeria innocua* DSM 20649, *Listeria monocytogenes* DSM 20600, *Pseudomonas aeruginosa* BFE 162 and *Staphylococcus aureus* S1 by agar spot method of Schillinger and Lücke (1989). Cultures were grown on the respective broth media for 24 h. Sterilised petri-plates were plated with MRS agar (containing 0.2 % glucose) and allowed to dry. These were spotted with a drop of the broth culture of the producer strain and incubated at 30°C for 24 h. The 0.1 ml of an overnight culture (~10^7 cells) of each indicator strain was inoculated into 7 ml of soft MRS agar (containing 0.7 % agar) and poured over the plate on which the producer was grown, respectively. These were incubated at 30°C for 24 h. After incubation, the plates were checked for inhibition zones (clearing of the medium) around the producer colony.

Bacteriocin activity of the LAB isolates was estimated using an agar spot assay as described by Schillinger *et al.* (1993).

Biogenic amine

The ability of LAB isolates to produce biogenic amines was determined qualitatively on an improved screening medium as described by Bover-Cid and Holzapfel (1999) using a 'cocktail' of four precursor amino acids (histidine, lysine, ornithine and tyrosine). Cultures previously grown and sub-cultured twice in biogenic amine sub-culturing medium were spotted onto the plates containing screening medium. Change of the bromocresol purple indicator to purple was considered as index of significant amino acid decarboxylase activity, corresponding to >350 mg of a particular amino acid/litre (Olasupo *et al.,* 2001).

Hydrophobicity assay

Bacterial adhesion to hydrocarbons was determined and results were expressed according to Tamang *et al.* (2009b). Fresh cultures were grown in MRS broth at 30ºC for 24 h and centrifuged at 8,000 *g* for 5 min. The pellet was washed three times with 9 ml of Ringer solution (Merck, Germany), and thoroughly mixed in a vortex. The 1 ml of the suspension was taken and the absorbance at 580 nm was measured. Then, 1.5 ml of the suspension was mixed with equal volume of n-hexadecane (RM 2238, HiMedia) in duplicates and mixed thoroughly in a vortex. The phases were allowed to separate for 30 min at room temperature, after which aqueous phase was carefully removed and absorbance at 580 nm was measured. Percentage hydrophobicity was expressed as follows: hydrophobicity % = $[A_0\text{-}A/A] \times 100$, where A_0 and A are the absorbance values of the aqueous phase before and after contact with n-hexadecane. The percent hydrophobic index greater than 70 % was arbitrarily classified as hydrophobic (Nostro *et al.,* 2004).

DPPH radical scavenging activity

1,1-diphenyl-2-picryl hydrazyl (DPPH) (Sigma, USA) radical scavenging activity was carried out in a 96 well microtitre plate following the method of Yamasaki *et al.* (1994). The hydrogen atoms or electrons donating ability of the corresponding extracts and some pure compounds were measured from the bleaching of purple coloured methanol solution of DPPH. A 10 µl aliquot of the extract (from 21 mg/ml to 21 µg/ml) was added to 200 µl of DPPH in methanol solution (100 µm) in a 96-well microtitre plate (Tarson Products (P) Ltd., India). The final concentration of the test and standard solutions used are 1000µg/ml, 500µg/ml, 250µg/ml and 125µg/ml. After incubation at 37°C for 30 min, the absorbance of each solution was determined at 490 nm using ELISA reader (Bio-Rad Laboratories Inc, USA, Model 550) against the corresponding test and standard blanks and the remaining DPPH was calculated. IC_{50} is the concentration of the sample required to scavenge 50% of DPPH free radicals.

ABTS radical scavenging activity

2, 2’-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) (Sigma, USA) radical scavenging activity was carried out in a 96 well microtitre plate following the method of Re *et al.* (1999). ABTS radical cation ($ABTS^{+}$) was produced by reacting ABTS stock solution (7 mM concentration) with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 12-16 h before use. To 0.2 ml of various concentrations of the extract or standards added, 1 ml of methanol and 0.16 ml of ABTS solution to make a final volume of 1.36 ml. After 20 min of the incubation in dark, the absorbance was measured at 734 nm using ELISA reader (Bio-Rad Laboratories

Inc, USA, Model 550). Blank is maintained without ABTS. IC_{50} is the concentration of the sample required to scavenge 50% of ABTS free radicals.

Total Phenol Content estimation

The total phenolic content (TPC) of the samples was estimated using the Folin-Ciocalteau micro-method described by Waterhouse (2005). The extract (60-300 µl) was diluted with deionised water to 4.8 ml and 300 µl of Folin-Ciocalteau Reagent was added and shaken. After 8 min, 900 µl of 20 % Sodium carbonate solution was added with mixing to neutralize it. The solution was allowed to stand at 40ºC for 30 min. before reading the absorbance using UV-VIS spectrophotometer (Analytik Jena, Germany) at 765 nm. Gallic acid (0-50 µg) was used as standard, and the results were reported as mg gallic acid equivalent per gram of fresh weight.

Statistics

The data were analysed by determining standard deviation (SD), standard error of measurement (SEM) and analysis of variance (ANOVA) (Snedecor and Cochran, 1989).

Results and Discussion

Amylolytic and proteolytic activities

All strains of *Bacillus*, previously isolated from *tungrymbai* 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 1). *Bacillus* strains except *B. subtilis* BK1:B18 from *bekang* showed protease activity (0.5–3.0 U/ml) (Table 1).

Table 1. Amylolytic and proteolytic activities and production of poly-glutamic acid (PGA) of/by *Bacillus* species isolated from *tungrymbai* and *bekang.*

Strain	α-Amylase[a] (U/ml)	Protease[a] (U/ml)	PGA production (mg/ml)
Tungrymbai			
Bacillus subtilis (n = 12)	3.7-5.7	1.3-3.2	0.8-2.8
B. licheniformis (n = 7)	1.8-2.9	1.1-1.3	0.9-2.6
B. pumilus (n = 6)	0	0.7-0.9	1.2-2.1
Bekang			
B. subtilis (n = 8)	2.1-4.8	1.8-3.0	1.8-2.8
B. licheniformis (n = 4)	2.4-3.2	1.3-2.4	0.8-1.3
B. pumilus (n = 4)	0	0.5-1.0	1.1-1.3

Contd.

B. sphaericus (n = 2)	0	0.8-1.0	0.9-1.0
B. coagulans (n = 2)	0.3-0.8	0.4-0.9	1.5-1.6
B. circuans (n = 2)	1.3-1.6	1.2-1.3	1.0-1.3
B. brevis (n = 3)	1.7-1.9	0.7-0.9	0.9-1.3
Control			
B. subtilis (natto) Miura strain[b]	ND	ND	3.6

[a]All strains showed >2.0 mm zones in starch and casein hydrolysis agar plate, respectively.

n, Number charges of strains tested.

[b]Reference strain

ND, not determined

The data represent the means of three set

Among bacilli, *B. subtilis* produced the highest proteolytic activity (Table 1). Garcia *et al.* (1994) found that proteinases obtained from *B. 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 and others 1971), improves the digestibility of proteins (Brenslaw and Kleyn, 1973). *B. licheniformis* and *B. coagulans* have been proven to produce thermostable α-amylase at alkaline pH (Medda and Chandra, 1980). Strains of LAB from *tungrymbai* and *bekang* did not show any amylolytic and proteolytic activity.

Enzymatic profiles

The commercial API-zym kit is used as a rapid and simple means of evaluating and localising 19 different hydrolases of microorganisms associated with dairy fermentations (Arora *et al.,* 1990). 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 (Table 2) produced by the *Bacillus* strains isolated from *tungrymbai* and *bekang* are possible traits of desirable quality for their use in production of typical flavour. All strains tested showed relatively moderate esterase (C4) and phoshpohydrolase activity (Table 2). However, they showed no detectable proteinase activity with the methods applied. Alkaline phospatase activity was exhibited moderately by all the strains of *Bacillus* tested. Alkaline phospatase dephosphorylates inactive derivatives of many antibiotics in the final step of biosynthesis and a direct relationship between intracellular enzyme level and antibiotics formation was well established (Majumdar and Majumdar, 1971).

Table 2 - Enzymatic profiles of *Bacillus* spp. isolated from *tungrymbai* and *bekang* using API-zym commercial kit.

Enzyme	Strain (Activity in nanomoles)														
	BME:B20	BK2:B8	BD1:B22	BT:B11	BME:B23	TS1:B25	TB1:B10	BK1:B18	BD1:B1	TP1:B1	TB2:B13	TM1:B12	BAV:B1	BME:B10	TP1:B5
Control (without enzyme)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alkaline phosphatase	10	10	5	5	5	10	5	5	5	5	0	10	10	5	0
Esterase (C4)	20	20	5	10	5	10	5	5	20	10	10	5	5	5	5
Esterase lipase (C8)	20	5	5	10	5	5	5	10	5	20	0	5	10	0	5
Lipase (C14)	0	0	0	0	0	0	0	0	5	0	5	0	0	5	5
Leucine arylamidase	20	5	0	10	5	0	10	5	≥40	20	0	10	0	5	0
Valine arylamidase	0	5	5	0	0	20	0	0	5	0	0	20	0	20	0
Cystine arylamidase	20	0	5	0	0	0	0	0	0	0	0	0	0	0	0
α-chymotrypsin	0	0	0	0	0	0	0	≥40	0	0	0	0	0	0	0
Acid phosphatase	5	5	≥40	5	5	5	30	5	10	5	0	0	0	0	0
Napthol-AS-BI-phosphohydrolase	5	5	5	5	5	20	5	5	5	5	5	5	5	5	5

Data represents the means of 3 sets of experiment.

Strains BME:B20 and BT:B11, *B. sphaericus*; BK2:B8 and BD1:B22, *B. circulans*; BME:B23, *B. brevis*; TS1:B25, BD1:B1 and TP1:B1, *B. subtilis*; TB2:B13, *B. pumilus*; TM1:B12, BAV:B1 and BME:B10, *B. licheniformis*; TP1:B5, *B. coagulans*

Trypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β- glucosaminidase, α-mannosidase, α-fucosidase were not hydrolyzed by any strain tested.

PGA-production by Bacillus

Poly-glutamic acid (PGA) is one of the few naturally occurring polyamides, 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 *chungkookjang* 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 strains of *Bacillus* previously isolated from *tungrymbai* and *bekang* (Chettri, 2012) was carried out (Table 1). *B. subtilis* TS1:B25 (*tungrymbai*) and *B. subtilis* BT:B9 (*bekang*) accounted for the highest production of PGA (2.8 mg/ml each) amongst the other strains tested (Table 1) which suggests that *B. subtilis* is the most potent PGA producer than the other species of *Bacillus*. The most striking feature of the γPGA produced by *B. subtilis* (*natto*) include its very large molecular mass of over 10^5 Da and the presence of both L- and D- glutamic acids (Saito and others 1974). *B. subtilis* and *B. licheniformis* are the most widely used industrial producers of γ-PGA (Stanley and Lazazzera, 2005). LAB previously isolated from *tungrymbai* and *bekang* were tested for their ability to degrade poly-glutamic acid (PGA) but none of the strains were able to degrade it.

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 *tungrymbai* and *bekang* were tested (data not shown). *E. faecium* TB1:L5 and TSB:L2 (*tungrymbai*) showed the lowest acidification value of pH 4.3, followed by *E. durans, E. faecium* and *E. cecorum* dropping the pH upto 4.4. 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. About 45% of LAB strains of *tungrymbai* and about 49% of LAB strains of *bekang* caused coagulation of milk at 30ºC with a significant drop in pH. All LAB strains coagulated skim milk (data not shown). Coagulation of milk by LAB strains shows their potential as starters or adjunct cultures in the production of fermented products.

Antimicrobial activities

Most of the LAB strains showed anitimicrobial activities against a Number charges of potentially pathogenic Gram-negative and Gram-positive bacteria (indicator strains), showing antagonism (data not shown). This reveals that antimicrobial properties of functional LAB can reduce the Number charges 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 isolated from *tungrymbai* and *bekang* could not produce bacteriocin under the applied condition. Production of bacteriocin depends on a Number charges of intrinsic and extrinsic factors including redox potential, water activity, pH and temperature (Delgado *et al.,* 2005).

Screening of Biogenic amines-producing LAB

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). Strains of LAB isolated from *tungrymbai* 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. The production of biogenic amines by LAB to be selected as starter cultures is not a desirable property (Buchenhüskes, 1993; Holzapfel, 1997).

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 (Ding and Lämmler, 1992; Vinderola *et al.,* 2004). A few strains of LAB isolated from *tungrymbai* and *bekang*, had more than 70% hydrophobicity (Table 3), indicating their hydrophilic nature. *E. faecium* TM2:L6 (*tungrymbai*) and *E. faecium* BAV:E2 (*bekang*) showed the highest degree of hydrophobicity of 72.7% and 71.6%, respectively (Table 3). 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).

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 (Holzapfel, 2002). Phytic acid has the strong ability to chelate multivalent metal ions, especially zinc, calcium, and iron (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 charges of foods of plant origin (Chavan and Kadam, 1989; Mbugua *et al.,* 1992). In *tungrymbai*, 34.5% 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 (data not shown). In *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 (data not shown). This proves that *tungrymbai* and *bekang* contains lowest level of phytic acid and oligosaccharides that make them fit for consumption.

Antioxidant capacity and total phenol content

Tungrymbai and *bekang* were tested for their antioxiadant capacity and total phenol content assessment (Table 4 and 5). A lower value of antioxidant activity as compared to the standard (Ascorbic acid, IC_{50} = 2.69 ± 0.0 µg/ml) in scavenging of stable free radical (DPPH) was found in samples of *tungrymbai* and *bekang* (Table 4 and 5). DPPH scavenging activity (IC_{50} values) of *tungrymbai* and *bekang* samples varied (Table 4 and 5). The highest DPPH scavenging activity of *tungrymbai* was shown by the sample collected from Shyrmang (516.7 ± 35.1 µg/ml) (Table 4), whereas in *bekang*, the highest value was recorded in sample collected from Ngopa (456.7 ± 30.6 µg/ml) (Table 5).

Table 3. Hydrophobicity of LAB strains from *tungrymbai* and *bekang*.

Strains	Hydrophobicity (%)
Tungrymbai	
Lb. brevis (n= 13)	10.5-71.0
E. cecorum (n= 7)	12.0-43.4
E. faecium (n= 19)	6.3-72.2
E. durans (n= 9)	4.8-24.7
E. raffinosus (n= 6)	3.5-62.8
E. hirae (n= 4)	7.5-24.3
Bekang	
E. cecorum (n= 7)	13.7-41.2
E. faecium (n= 24)	23.1-71.6
E. durans (n= 9)	4.3-21.8
E. raffinosus (n= 6)	2.4-22.8
E. hirae (n= 7)	9.5-21.9

n, Number charges of strains tested.

The data represent the means of three set

ABTS radical scavenging activity of the samples of *tungrymbai* and *bekang* were found to be lower than that of the standard (Ascorbic acid, IC_{50} = 11.25 ± 0.4 µg/ml) used. ABTS scavenging activity (IC_{50} values) of *tungrymbai* and *bekang* samples collected from different places varied and ranged between 243.3±40.4 to 153.3±25.2 µg/ml (*tungrymbai*, Table 4) and 170.0±30.0 to 145.0±22.2 µg/ml (*bekang*, Table 5). The highest value of total phenolic content (TPC) in *tungrymbai* sample collected from Shyrmang was 3.7±0.1 mg GAE/g fresh weight and in *bekang* sample collected from Ngopa was 4.2±0.3 mg GAE/g fresh weight (Table 4 and 5). Wang and others (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.

Table 4. Antioxidant capacity and total phenol content estimation of *tungrymbai.*

Place of collection in Meghalaya	DPPH	ABTS	TPC
Police bazaar (n = 3)	773.3 ± 30.6^{a}	243.3 ± 40.4^{a}	2.4 ± 0.1^{ad}
Mylliemgat (n = 4)	706.7 ± 35.2^{ad}	205.7 ± 15.3^{b}	2.5 ± 0.3^{ac}
Sohra (n = 3)	690.0 ± 20.0^{bcd}	203.3 ± 25.2^{bc}	2.1 ± 0.1^{bcde}
Nongpyuir (n = 5)	723.3 ± 30.6^{ac}	183.3 ± 25.2^{cd}	2.6 ± 0.3^{ab}
Saldew (n = 3)	663.3 ± 25.2^{bd}	156.7 ± 15.3^{e}	2.2 ± 0.1^{ae}
Shyrmang (n = 3)	516.7 ± 35.1^{e}	153.3 ± 25.2^{ef}	3.7 ± 0.1^{a}

DPPH = 1, 1-diphenyl-2-picryl hydrazyl, IC_{50} values ± SD (µg/ml); ABTS = 2, 2'-azino-bis-(3-ethylbenzo-thiazoline-6-sulfonic acid), IC_{50} values ± SD (µg/ml); TPC = Total phenol content, mg gallic acid equivalent/g fresh weight

n, Number charges of samples tested. Data represents the means (± SD).

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

Standard (Ascorbic acid) values for DPPH is 2.69 ± 0.0 µg/ml and ABTS is 11.25 ± 0.4 µg/ml

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). Pourmorad *et al.,* (2006) reported that the extract which contained highest amount of flavonoid and phenolic compounds, exhibited the greatest antioxidant activity. Moktan *et al.,* (2008) reported that the TPC of *kinema* fermented with *B. 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 *tempe* inoculated with *Aspergillus oryzae*, *B. natto* and *Rhizopus oligosporous*, respectively, have proved to be more stable against lipid peroxidation than steamed soybeans (Nagai and Tamang, 2010). Methanolic extracts of *thua-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). *Chungkukjang* (a traditional Korean fermented soyfood) made by fermenting large black soybean 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 scavenging activity may not show DPPH scavenging activity. All these reports firmly support the antioxidant potentials of the extracts of *tungrymbai* and *bekang*, observed in the present study is due to the presence of phenolic compounds.

Table 5. Antioxidant capacity and total phenol content estimation of bekang.

Place of collection in Mizoram	DPPH	ABTS	TPC
Thakthing bazaar (n = 4)	480.0 ± 20.0[a]	166.7 ± 25.2[ac]	2.6 ± 0.1[b]
Serchhip (n = 4)	493.3 ± 25.2[a]	145.0 ± 22.2[beg]	4.1 ± 0.2[a]
Kolasib (n = 3)	476.7 ± 45.1[a]	160.0 ± 30.0[afg]	3.9 ± 0.1[a]
Melthum (n = 5)	486.7 ± 45.1[a]	163.3 ± 41.6[ade]	3.6 ± 0.9[a]
Saitual (n = 3)	470.0 ± 20.0[a]	148.3 ± 28.4[bcdf]	4.1 ± 0.2[a]
Ngopa (n = 3)	456.7 ± 30.6[a]	170.0 ± 30.0[a]	4.2 ± 0.3[a]

DPPH = 1, 1-diphenyl-2-picryl hydrazyl, IC_{50} values ± SD (µg/ml); ABTS = 2, 2'-azino-bis-(3-ethylbenzo-thiazoline-6-sulfonic acid), IC_{50} values ± SD (µg/ml); TPC = Total phenol content, mg gallic acid equivalent/g fresh weight.

n, Number charges of samples tested; Data represents the means (± SD).

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

Standard (Ascorbic acid) values for DPPH is 2.69 ± 0.0 µg/ml and ABTS is 11.25 ± 0.4 µg/ml

Conclusion

Tungrymbai and *bekang* are naturally fermented ethnic soybean foods of North East India. Scientific knowledge on these products is unknown outside the North East region of India. This study revealed that strains of bacilli along with LAB 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 bactericion 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, and enhancement of antioxidant activity.

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