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Short communication

Bacillus species isolated from *tungrymbai* and *bekang*, naturally fermented soybean foods of India

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ABSTRACT

Tungrymbai and *bekang* are naturally fermented soybean foods commonly consumed in Meghalaya and Mizoram states of India. A total of 39 samples of *tungrymbai* and 43 samples of *bekang* were collected from different villages and markets of Meghalaya and Mizoram, respectively and were analysed for microbial load. In both *tungrymbai* and *bekang*, the average population of *Bacillus* spp. was 8.2 ± 0.1 log cfu/g. A total of 428 isolates of *Bacillus* were isolated from *tungrymbai* (211) and *bekang* (217) for detailed identification. On the basis of a combination of phenotypic and molecular characterisation using ARDRA, ITS-PCR and RAPD-PCR techniques, species of *Bacillus* isolated from *tungrymbai* were identified as *Bacillus licheniformis* (25.5%), *Bacillus pumilus* (19.5%) and *Bacillus subtilis* (55%), and species of *Bacillus* from *bekang* were *Bacillus brevis* (2%), *Bacillus circulans* (7.5%), *Bacillus coagulans* (6.5%), *B. licheniformis* (16.5%), *B. pumilus* (9.1%), *Bacillus sphaericus* (4.6%), *B. subtilis* (51.8%), and *Lysinibacillus fusiformis* (2%). The most dominant bacterium in both products was *B. subtilis*.

1. Introduction

Consumption of naturally fermented soybeans is common among the different ethnic groups of people in the Oriental countries including the Himalayan regions of India, Nepal and Bhutan (Tamang, 2010). *Bacillus*-fermented sticky soybean foods of Asia are *natto* of Japan (Kubo et al., 2011), *chungkokjang* of Korea (Shin et al., 2012), *kinema* of India, Nepal and Bhutan (Tamang, 2003), *aakhone*, *hawaijar* and *peruyaan* of India (Tamang et al., 2012), *thua nao* of Thailand (Inatsu et al., 2006), *pepok* of Myanmar, and *sieng* of Cambodia and Laos (Nagai and Tamang, 2010).

Tungrymbai is a naturally fermented soybean food eaten as a side-dish in Meghalaya state of India (Tamang et al., 2009). During its preparation, local varieties of soybean seeds are washed, soaked for about 4–6 h, the outer skin is removed by rubbing gently between the palms and is cooked (1–2 h) until all the water is absorbed and the soybeans can be pressed easily. The cooked beans are allowed to cool, and are packed with leaves of *Clinogyne dichotoma* or *Phrynium pubinerve* lined in the bamboo basket and covered by a thick cloth or jute bag. The covered basket is kept near the fireplace (25–40 °C) for natural fermentation for 3–5 days to get a sticky product.

Bekang is a naturally fermented, sticky soybean food of Mizoram state in India (Tamang et al., 2009). Small sized, yellow variety of soybeans are soaked overnight, and boiled for 1–2 h until the soybeans are cooked. The cooked soybeans are then spread on a bamboo basket/tray lined with the leaves of *Callicarpa arborea* or leaves of *P. pubinerve*. Little amount of firewood ash is added. The bamboo basket/tray containing the cooked soybeans is then covered with the same leaves, again covered with a soft cloth, and kept near the fireplace (25–40 °C) to ferment naturally for 3–4 days.

Bacillus is the dominant bacterium in many sticky fermented foods of Asia (Meerak et al., 2007; Tamang et al., 2002). Species of *Bacillus* isolated from *kinema* include *Bacillus circulans*, *Bacillus licheniformis*, *Bacillus sphaericus*, *Bacillus subtilis* and *Bacillus thuringiensis* (Sarkar et al., 1994, 2002; Tamang, 2003), however, *B. subtilis* is the dominant functional bacterium in *kinema* (Sarkar and Tamang, 1994; Tamang and Nikkuni, 1996). *Bacillus cereus*, *B. licheniformis* and *B. subtilis* were isolated from *hawaijar* of Manipur state of India (Jeyaram et al., 2008). *Bacillus amyloliquefaciens*, *B. licheniformis*, *Bacillus megaterium*, and *B. subtilis* subsp. *chungkokjang* were isolated from *chungkokjang*, naturally fermented soybean food of Korea (Kwon et al., 2009; Nam et al., 2012; Tamang et al., 2002). Japanese *natto* is the only *Bacillus*-fermented soybean food which is now produced by commercial mono-culture starter *B. subtilis* var. *natto* (Nishito et al., 2010). *B. subtilis* is a functional bacterium in Thai *thua nao* (Inatsu et al., 2006). *Bacillus* species have also been isolated from ethnic non-salted fermented locust bean (*Parkia biglobosa*) foods of Africa such as *dawadawa*, *iru* and *soumbala* which include *B. amyloliquefaciens*, *Bacillus atrophaeus*, *Bacillus badius*, *B. cereus*, *Bacillus firmus*, *Bacillus fumus*, *B. licheniformis*, *B. megaterium*, *Bacillus mojavensis*, *Bacillus mycoides*, *Bacillus pumilus*, *B. subtilis*, *B. sphaericus*, and *B. thuringiensis* (Ahaotu et al., 2013; Azokpota et al., 2006; Meerak

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Table 1
pH and microbial load of naturally fermented soybean foods of India.

Food	pH	Log cfu/g sample			
		Bacillus	LAB	Yeast	TVC
Tungrymbai of Meghalaya (n = 39)	7.4 (6.7–8.1) ± 0.2	8.2 (7.8–8.9) ± 0.1	4.1 (3.5–4.8) ± 0.1	2.8 (1.9–3.3) ± 0.2	8.9 (8.6–9.3) ± 0.1
Bekang of Mizoram (n = 43)	7.1 (6.8–7.5) ± 0.1	8.4 (7.8–8.7) ± 0.1	4.0 (3.2–5.4) ± 0.1	3.1 (2.3–3.6) ± 0.1	8.9 (8.5–9.4) ± 0.1

n, number of samples collected from different places of Meghalaya and Mizoram in India.
LAB, lactic acid bacteria; TVC, total viable count. Filamentous mould was not detected.
Data represents the means (±SD) of number of samples collected. Mean pH (±SD) of each sample is shown in parenthesis.

et al., 2008; Ouoba et al., 2010). The aim of this paper is to isolate and identify the predominant species of *Bacillus* in naturally fermented *tungrymbai* and *bekang* of India. These species were characterised using the molecular techniques such as amplified ribosomal DNA restriction analysis (ARDRA), internal transcribed spacer (ITS)-PCR and random amplified polymorphic DNA (RAPD)-PCR.

2. Materials and methods

2.1. Samples

A total of thirty-nine samples of *tungrymbai* and forty-three samples of *bekang* were collected from different villages and markets of

Table 2
Grouping of *Bacillus* based on the restriction profiles of ARDRA.

Sample	Strain	Putative species[a]	Restriction profile			ARDRA group	Species[b]
			*Hinf*I	*Rsa*I	*Cfo*I		
Bekang	BAV:B1	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BD1:B1	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BK1:B15	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BK1:B24	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BK2:B6	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BME:B10	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BME:B20	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BME:B23	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BT:B3	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BT:B9	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BT:B17	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BT:B20	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB1:B10	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB1:B16	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB1:B17	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB2:B8a	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB2:B8b	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB2:B11	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TB2:B13	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TM1:B1	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TP1:B4a	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TP1:B4b	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TM1:B5	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TM1:B12	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TP1:B1	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TS1:B6	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TS2:B13	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Tungrymbai	TSA:B15	*B. subtilis*	II	IV	VI	Group I	*B. subtilis*
Bekang	BK1:B13	*B. licheniformis*	II	IV	VII	Group II	*B. licheniformis*
Bekang	BT:B11	*B. licheniformis*	II	IV	VII	Group II	*B. licheniformis*
Tungrymbai	TP1:B2	*B. licheniformis*	II	IV	VII	Group II	*B. licheniformis*
Tungrymbai	TP1:B15	*B. licheniformis*	II	IV	VII	Group II	*B. licheniformis*
Tungrymbai	TP1:B5	*B. licheniformis*	II	IV	VII	Group II	*B. licheniformis*
Tungrymbai	TP2:B10	*B. licheniformis*	II	IV	VII	Group II	*B. licheniformis*
Bekang	BAV:B12	*B. cereus*	I	III	V	Group III	*B. cereus*
Bekang	BK2:B8	*B. cereus*	I	III	V	Group III	*B. cereus*
Bekang	BK2:B12	*B. cereus*	I	III	V	Group III	*B. cereus*
Bekang	BME:B6	*B. cereus*	I	III	V	Group III	*B. cereus*
Tungrymbai	TM2:B6	*B. cereus*	I	III	V	Group III	*B. cereus*
Tungrymbai	TS1:B25	*B. cereus*	I	III	V	Group III	*B. cereus*
Tungrymbai	TS2:B24	*B. cereus*	I	III	V	Group III	*B. cereus*
Tungrymbai	TSA:B4	*B. cereus*	I	III	V	Group III	*B. cereus*
Tungrymbai	TSB:B17	*B. cereus*	I	III	V	Group III	*B. cereus*
Bekang	BK1:B18	*B. cereus*	I	IV	V	Group IV	*B. cereus*
Bekang	BAV:B3	*B. subtilis*	I	IV	VII	Group V	*L. fusiformis*
Bekang	BAV2:B6	*L. fusiformis*	I	IV	VII	Group V	*L. fusiformis*
Bekang	BT2:B18	*L. fusiformis*	I	IV	VI	Group VI	*L. fusiformis*
Bekang	BAV:B15	*B. coagulans*	I	III	VI	Group VII	*B. subtilis*

[a] Identified by using the API-CHB kit.
[b] Species determined based on the homology identities of 16S rDNA sequences.

Meghalaya and Mizoram in India, respectively. Samples were collected aseptically in sterile bottles kept in an ice-box, transported to the laboratory, stored at 4 °C, and analysed within a week.

2.2. Microbiological analysis

Ten grams of sample was homogenised with 90 mL of 0.85% (w/v) sterile physiological saline in a stomacher lab-blender (400, Seward, UK) for 1 min. A serial dilution (10^{-1} to 10^{-8}) in the same diluent was made. Spore-forming bacilli were isolated by plating on nutrient agar (MM012, HiMedia, India), after the inactivation of vegetable cells by heating at 100 °C for 2 min (Tamang and Nikkuni, 1996) and then incubated at 37 °C for 24 h. Lactic acid bacteria (LAB) were isolated on plates of MRS agar (M641, HiMedia, India) supplemented with 1% $CaCO_3$ and incubated at 30 °C in an anaerobic gas-jar (LE002, HiMedia, India) for 48–72 h. The presence of filamentous fungi and yeasts was examined on potato dextrose agar (M096, HiMedia, India) and yeast-malt (YM) agar (M424, HiMedia, India), supplemented with 10 IU mL^{-1} benzylpenicillin and 12 μg mL^{-1} streptomycin sulphate, respectively, and which were incubated aerobically at 28 °C for 72 h. Total viable counts were determined on plate count agar (M091A, HiMedia, India) incubated at 30 °C for 48–72 h. Isolated colonies based on colony morphology were selected randomly from the highest diluted plates. Isolated bacteria were preserved in nutrient broth using 15% (v/v) glycerol at −20 °C.

2.3. Percent occurrence of Bacillus in samples

The occurrence of different species of *Bacillus* in samples was calculated out of total *Bacillus* isolates, and expressed in percentage.

2.4. Phenotypic characterisation

Cell morphology of all isolates and their motility were determined using a phase contrast microscope (Olympus CH3-BH-PC, Japan). Isolates were Gram-stained and tested for catalase production, and were preliminarily identified based on the phenotypic properties such as carbon dioxide production from glucose, ammonia production from arginine, growth at different temperatures as well as the ability to grow in different concentrations of sodium chloride and pH in nutrient broth (M002, HiMedia, India) following the method of Schillinger and Lücke (1987). Voges–Proskauer test, nitrate reduction, starch hydrolysis, casein hydrolysis, citrate utilization test, bile salt tolerance and anaerobic growth were determined following the methods of Claus and Berkeley (1986) and Duc et al. (2004). Fermentation and assimilation of carbon compounds were determined using API 50 CHB kits (BioMerieux, Basingstoke, UK) according to the manufacturer's instruction and results were analysed using the API software (APIWeb, Biomerieux). For identification of *Bacillus*, taxonomic key of Slepecky and Hemphill (2006) was followed.

2.5. Genotypic identification

2.5.1. Amplification of PCR

Bacterial genomic DNA was isolated according to the method of Zhang et al. (2002). Amplified 16S rDNA was obtained from each strain by Polymerase Chain Reaction (PCR) with the following universal primers; forward 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse 5′-AAGGAGGTGATCCAGCCGCA-3′ (Weisburg et al., 1991).

2.5.2. ARDRA

The amplified ribosomal DNA restriction analysis (ARDRA) technique using restriction enzymes was carried out with *Hinf*I (R6205, Promega), *Cfo*I (R6241, Promega) and *Rsa*I (Promega) (Jeyaram et al., 2010).

2.5.3. ITS-PCR analysis

The amplification of 16S–23S rDNA intergenic transcribed spacer (ITS) region was carried out in a 25 μL reaction mixture with 2 μL of cell free DNA lysate containing 30 μg of DNA, 2.5 μL of 10× PCR reaction buffer, 2.5 mM $MgCl_2$, each dNTP at a final concentration of 200 μM, each primer (16-1A: 5′-GAATCGCGCTAGTAATCG-3′ and 23-1B: 5′-GGGTTCCCCCATTCGGA-3′) (Tilsala-Timisjärvi and Alatossava, 1997) at a final concentration of 2.4 pmol/μL and 1.5 U of Taq DNA polymerase.

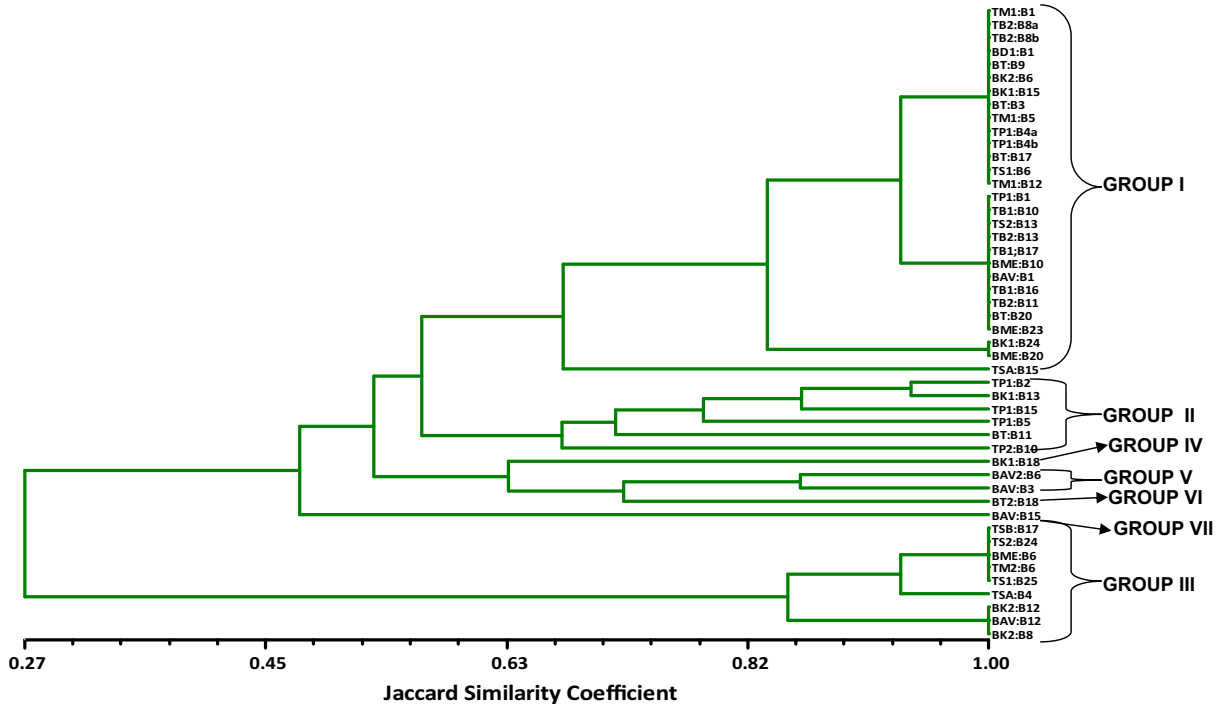

Fig. 1. Dendogram based on the UPGMA of Jaccard Similarity Coefficient (S_J) of combined ARDRA and ITS profiles of various isolates of *Bacillus* from *tungrymbai* and *bekang*. Groups I and VII belong to *B. subtilis* group, group II represents *B. licheniformis* group, groups III and IV belong to *B. cereus*, and groups V and VI belong to *Lysinibacillus fusiformis* group.

Amplification was started with an initial denaturation at 94 °C for 10 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and primer extension at 72 °C for 1 min with a final extension of 72 °C for 10 min. 5 μL of PCR amplified ITS products was analysed using 1.5% agarose gel.

2.5.4. RAPD-PCR analysis

The primers OPD18: 5′-GAGAGCCAAC-3′; OPN13: 5′-AGCGTCACTC-3′; E11: 5′-CTGGCTTGGTGTATGT-3′; OPD-05: 5′-TGAGCGGACA-3′; and M13: 5′-GAGGGTGGCGGTTCT-3′ (Pulido et al., 2005) were used for RAPD-PCR to differentiate at strain level.

2.6. Gel electrophoresis

The amplified DNA fragments were separated through gel electrophoresis by applying 10 μL of each PCR product with 1.5 μL of loading dye {(6×), DV4371, Promega, USA} into the wells of 1.5% agarose (V3125, Promega) gel containing 1.5 μL/mL Ethidium Bromide (Etbr) (H5041, Promega). DNA size markers (RMBD135, Genei; G5711, Promega) were added as standard for the calculation of size of the DNA fragments. The gel was run in 0.5× TBE buffer (89 mM/L Tris (H5131, Promega), 69 mM Boric acid (H5003, Promega), 25 mM Na_2EDTA (H5031, Promega, pH 8.0)) for 2 h at 80 V (Subcell GT, Biorad, USA) and photographed using gel documentation system (GelDoc FQ, Biorad, USA).

2.7. 16S rDNA sequence analysis

The sequencing reactions were performed using ABI PRISM 3100 Genetic Analyzers (Applied Biosystems) in both directions 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, India). 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 the GenBank database using BLAST and the Ribosomal Database Project (RDP).

2.8. Phylogenetic analysis

The ITS-PCR and RAPD-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 (S_J) and the un-weighed pair group method using arithmetic averages (UPGMA). To determine the closest known relatives of the partial 16S rDNA sequences obtained, nucleotide database searches were performed in NCBI GenBank and Ribosomal Database Project (RDP) release 10 using BLAST and SEQmatch programmes, respectively.

2.9. pH

The pH of the samples (10 g) was determined directly using a digital pH meter (Type 361, Systronics, India) calibrated with standard buffer solutions (Merck), after homogenisation in 20 mL of carbon-dioxide free distilled water.

3. Results and discussion

A total of 39 samples of *tungrymbai* and 43 samples of *bekang* were analysed for microbiological populations (Table 1). In both *tungrymbai* and *bekang*, the average population of *Bacillus* spp. was 8.2 ± 0.1 log cfu/g, population of LAB was in 4.1 ± 0.1 log cfu/g, load of yeasts was 2.8 ± 0.2 log cfu/g, and total viable count was 8.9 ± 0.1 log cfu/g, respectively. Filamentous fungi were not detected in any market sample of *tungrymbai* and *bekang*. The mean pH value of *tungrymbai* and *bekang* was 7.4 ± 0.2 and 7.1 ± 0.1, respectively (Table 1).

Due to the dominance (>10^7 log cfu/g) of *Bacillus* in *tungrymbai* and *bekang*, only *Bacillus* strains were isolated and characterised in this study. A total of 428 isolates (211 isolates from 39 *tungrymbai* samples and 217 isolates from 43 *bekang*) were selected by their distinct colony morphologies, their ability to grow in anaerobic agar, hydrolysis of starch and arginine, catalase, growth at different temperatures, pH and sugar fermentation (data not shown). The ARDRA patterns of all restriction enzymes were combined to achieve different ARDRA groups and randomly selected 48 strains of *Bacillus* spp. were grouped into 7 groups (Table 2). However, there are certain limitations to this ARDRA assay, such as the use of universal primers than *Bacillus*-specific primers for PCR amplification. Thus the subsequent species identification was relied on prior genus identification by phenotypic and biochemical methods. The tentative identification by using API 50 CHB was in good concordance with those by the genetic identification, that is, the strains of *B. subtilis* were discriminated into group I (exception of group VII for strain BAV:B15), those for *B. licheniformis* were grouped as group II, those for *B. cereus* were discriminated into two groups, groups III and IV, and those for *Lysinibacillus fusiformis* were discriminated into two groups, groups V and VI based on ARDRA (Table 2).

ITS-PCR profile clearly differentiated 7 groups of ARDRA into 16 subgroups of ITS (data not shown). ITS region was reported for exhibiting a higher interspecies variation than rRNA genes without intraspecies variation in *Bacillus* (Ouoba et al., 2004). The ARDRA and ITS-PCR profiles (banding patterns) were scored manually and dendrogram was prepared (Fig. 1). 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 belong to *L. fusiformis* group. Groups V and VI were misidentified as *B. sphaericus* during phenotypic identification. *L. fusiformis* is recovered only from the samples of *bekang* and not from *tungrymbai*. RAPD profiles of major *B. subtilis* group of isolates from *tungrymbai* and *bekang* were also applied for confirmation of *B. subtilis* (data not shown).

Based on the detailed characterisations and molecular identification profiles, three species of *Bacillus* — *B. licheniformis*, *B. pumilus* and *B. subtilis*, isolated from *tungrymbai* samples were identified. Similarly, seven species of *Bacillus* isolated from *bekang* samples were identified as *Bacillus brevis*, *Bacillus coagulans*, *B. circulans*, *B. licheniformis*, *B. pumilus*, *B. sphaericus*, *B. subtilis* along with *L. fusiformis*. The occurrence of *B. subtilis* in *tungrymbai* was 55% (out of 211 total isolates) which was the most dominant bacterium, followed by *B. licheniformis* (25.5%) and *B. pumilus* (19.5%). Similarly, in *bekang B. subtilis* was 51.8% (out of 217 isolates), *B. licheniformis* (16.5%), *B. pumilus* (9.1%), *B. brevis* (2%), *B. circulans* (7.5%), *B. coagulans* (6.5%), *B. sphaericus* (4.6%), and *L. fusiformis* (2%).

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