

Original Research Article

<http://dx.doi.org/10.20546/ijcmas.2017.602.124>

Identification and Characterization of *Lactobacillus* isolates from fermented soya food “Tungrymbai”, Meghalaya, India

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ABSTRACT

Keywords

Tungrymbai,
Lactobacillus,
Soy foods, API,
NCBI, 16SrDNA.

Article Info

Accepted:
20 January 2017
Available Online:
10 February 2017

Tungrymbai is one of the most popular and ethnic fermented food of Garo and Khasi tribes of Meghalaya region, produced by fermentation of soya beans by heterogeneous Lactic Acid Bacteria. To assess the nature of microbes and their source during spontaneous fermentation is generally studied to explore the microbial diversity of this particular food. In this era, lactic acid bacteria has drawn maximum attention in food and nutrition science due to their nutraceutical potential producing certain biologically active peptides, along with other functional and probiotic attributes and most importantly having GRAS status. In the present investigation, isolates were screened on MRS medium, *Lactobacillus* strains were identified by phenotypic (Gram staining, catalase activity and API test) and genotypic by molecular characterization (PCR). Phylogenetic tree of the most closely related *Lactobacillus* species has been constructed by using MAFFT sequence alignment tool. Sequences are deposited in GeneBank and NCBI bearing the accession number.

Introduction

Tungrymbai is an ethnic fermented indigenous soya food popular to the Khasi and Garo tribes of Meghalaya. *Lactobacillus* along with other lactic acid bacteria's are its natural habitat (Thockhom and Joshi, 2012). *Tungrymbai* is a naturally fermented soybean food eaten as a side dish in Meghalaya state of India (Tamang *et al.*, 2009). It forms an intricate part of the diet and serves as a cheap source of high protein food in local diet. The protein content in *Tungrymbai* has been found to be 45.9 g % on a dry weight basis. Whereas, fat, fibre and ash have been found

to be 30.2, 12.8 and 5.5 g % respectively (Agrahar-Murungkar and Subbulakshmi, 2006). It is a sticky food that exhibits its unique flavor and texture that may not be palatable to everyone. 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.

Proper identification and characterization of lactobacilli is not only done by phenotypic methods but it includes molecular studies as well. Molecular studies focuses on DNA sequencing and sequence analysis of evolutionary stable genes to study bacterial phylogeny and diversity (Tringe and Hugenholtz, 2008). For this purpose, genes that code for the 5S, the 16S, the 23S rRNA and spaces between these genes are supposed to be potential candidates. Among these the most common part of the DNA used for taxonomic purposes is the 16S rRNA gene (Harmsen and Karch, 2004; Tortoliet *al.*, 2003; Palysset *al.*, 1997) due to its highly conserved nature among three regions, (Clarridgeet *al.*, 2004) hence, the 16S rRNA gene is universal in bacteria, and so relationships can be measured among all bacteria (Woese and Stackebrandt, 1985; Woeseet *al.*, 1987). Major application of 16S sequence analysis is identification, classification and estimation of bacterial diversity of isolated pure culture along with environmental samples without culturing through metagenomic approaches (Rajendhran and Gunasekarsan, 2011).

Different studies on probiotic potential of *Lactobacillus* has been conducted previously focusing on its isolation, acid and bile tolerance and antimicrobial properties but molecular level study of isolates from fermented soy foods has not been explored yet. Keeping in view the potential health benefits and nutraceutical properties of probiotics, this study is designed to explore novel lactic acid bacteria, particularly, *Lactobacillus* sp. from ethnic fermented soya food, “*Tungrymbai*” up to molecular level

characterization along with phylogenetic studies. In Meghalaya, this study will help to provide a more valuable functional food with particular health benefits. The aim of this paper is to isolate and identify the predominant species of *Lactobacillus* in naturally fermented *Tungrymbai* of Meghalaya, India. These species were characterized using phenotypic and molecular techniques for confirmation of genus and species level of *Lactobacillus* strains, along with DNA sequencing and analysis of phylogenetic studies by utilizing 16S rRNA gene.

Materials and Methods

Sample collection and bacterial growth enrichment

Homemade and commercial samples of fermented soya (*Tungrymbai*) were collected from different region of Meghalaya state and included in this investigation (Table 1). The enrichment process was carried out by inoculating approximately 1 ml of a mix of the liquor and poured into 50 ml sterile MRS broth (HiMedia, India) and incubated at 37°C for (2-5) days (Abbas and Mahasneh, 2014). All samples were collected into sterile glass bottles and were kept in the laboratory at refrigeration temperature (4-6°C) for further analysis.

Identification of bacterial strains

All isolates were tested for catalase activity, Gram reaction and cell morphology (Guessas and Khal, 2004). The identification of strains was performed according to their morphological, cultural and biochemical properties based on their specific characteristics as described in Bergy’s manual (Buchanan and Gibbons, 1974). The strains were tested for the production of acids from carbohydrates and related compounds using API 50 CH kits (HiMedia, India) according to

the manufacturer's instructions. Results were scored after incubation at 37°C for 24 and 48 hours. These results were put on the apiweb™ identification software with database (V5.1) which uses the phenotypic data to predict a species identity. Interpretations of the fermentations profiles were facilitated by comparing all results obtained for the tested isolates with information from the computer aided database, apiweb™ (<https://apiweb.biomerieux.com>).

Confirmation of lactobacilli isolates by colony PCR

Molecular characterization of isolates was done by Polymerase Chain Reaction (PCR) using primers (27F and 1492R) (Table 2). Template was prepared by picking freshly grown colony and transferred to TE buffer and incubate at 80°C for 15 minutes. This was further amplified by PCR and confirmed by running product on agarose gel (1%) in gel electrophoresis. PCR mixture was initially heated at 94°C for 5 minutes followed by cycles of denaturation at 94°C for 1 min, annealing 56°C for 1 min and extension was performed at 72°C for 5 minutes.

Results and Discussion

This study was conducted to isolate *Lactobacillus* strains from ethnic fermented soya food "*Tungrymbai*" from the various places of Meghalaya (North-eastern region of India) and explore their phenotypic and genotypic characteristics for further, development of value added products by identifying productive microbial strains.

Phenotypic characterization of *Lactobacillus* strains

A total of three strains were isolated from the various samples of *Tungrymbai* obtained from different places of Meghalaya, India (Table

1). Three catalase negative and Gram positive bacteria were isolated from fermented soya foods and considered as presumptive LAB (Figure 1). Further, biochemical tests of all the isolates were carried out by API CH 50 Microbial Identification kit (*bioMerieux, India*) through sugar fermentation pattern, ammonia production from arginine, gas production from glucose were carried out for the initial characterisation of lactic acid bacteria isolated from the samples.

From the table 3, the isolates, both RD10 and K20 showed negative whereas the isolate, K38A showed positive results for D-mannitol, D-mannose, Methyl- α -D-mannopyranoside, Amygdalin, Arbutin, Salicin, D-lactose, D-raffinose, D-celiobiose, Gentibiose respectively. RD10 and K38A showed positive whereas K20 showed negative results for tests like L-arabinose, D-ribose, and D-galactose. K20 and K38A showed positive whereas RD10 showed negative results for D-maltose, D-saccharose, D-glucose, and D-trehalose respectively. K20 and K38A showed negative whereas RD10 showed positive for D-xylose, potassium gluconate, potassium-2-ketogluconate, and potassium-5-ketogluconate respectively. All the three isolates were able to utilize esculin ferric citrate, n-acetyl glucosamine, D-fructose, resulting to be positive and were not able to utilize glycerol, erythritol, D-arabinose, L-Xylose, D-Adonitol, Methyl- β -D-Xylopyranoside, D-sorbitol, L-Sorbose, L-Rhomnose, Dulcitol, Inositol, Inulin, Methyl α -D-Glucopyranoside, D-Turanose, D-Lyxose, D-Tagatose, D-Fucose, L-Fucose, D-Arbitol, L-Arbitol thereby resulting to be negative respectively (Table 3).

Hence, from the above biochemical tests it was assumed that all the three isolates belonged to the group of heterofermentative lactic acid bacteria. The tentative identification by using API 50 CH was in

good concordance with those by the genetic identification which later on showed that RD10 and K38A belongs to *Lactobacillus brevis* and K20 belongs to *Lactobacillus fermentum* (Figure 2).

Molecular confirmation and 16S rDNA sequence analysis of *Lactobacillus* strains

Easiest and simplest way for identification of LAB is amplification of 16S rRNA, 16S-23S intergenic spacer region (ISR), or 23S rRNA universal gene by designing specific primers (Kimet *al.*, 2005). In the present study, primer 27F and 1492R were used for amplification conserved regions of 16S rRNA, resulted in product of 1.5kb fragments confirming that

the isolate bacteria was from genus *Lactobacillus*. Rahayu *et al.*, 2009 used same primers set for amplification of bacterial 16S rRNA gene and reported the PCR product of 1.5kbs. The electrophenogram data for 16S rDNA sequence was validated using Chromas 2.33 software. Sequences obtained were matched with previously published bacterial 16S rDNA sequences available in the GenBank database using BLAST. The sequences determined in this study have been deposited in the NCBI GenBank database with accession numbers (Table 4).

The FASTA sequences of the identified strains after 16S rDNA sequence analysis are as follows:

16s rRNA sequence of *Lactobacillus brevis* (GeneBank Accession no.KX572212)

```
>gi|1044329733|gb|KX572212.1| Lactobacillus sp. strain brevis  
RD10 16S ribosomal RNA gene, partial sequence  
AGCTTCCGTTGAATGACGTGCTTGCCTGATTTTAAACAATGAAGCGAGTGGCGAACTGGTGAGT  
AACACGTGGGAAATCTGCCCAGAAGCAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATA  
ACAACAAAGTCCGCATGGATTTTGTGTTGAAAGGTGGCTTTCGGCTATCACTTCTGGATGATCCCG  
CGGCGTATTAGTTAGTTGGTGAGGTAAAGGCCACCAAGACGATGATACGTAGCCGACCTGAGA  
GGTAATCGGCCACATTGGGACTGAGACACGGCCAACTCCTACGGGAGGCAGCAGTAGGGAA  
TCTTCCACAATGGACGAAAGTCTGATGGAGCAATGCCGCGTGAGTGAAGAAGGGTTTCGGCTCG  
TAAACTCTGTTGTTAAAGAAGAACACCTTTGAGAGTAACTGTTCAAGGGTTGACGGTATTTAA  
CCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCC  
GGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTTTTAAAGTCTGATGTGAAAGCCTTCGGCTT  
AACC GGAGAAGTGCATCGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGT  
GTAGCGGTGGAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTAGTCTGTA  
ACTGACGCTGAGGCTCGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGCCATGCCGT  
AAACGATGAGTGCTA
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16s rRNA sequence of *Lactobacillus fermentum* (GeneBank Accession no.KU644579)

```
>gi|999867666|gb|KU644579.1| Lactobacillus fermentum strain K20  
16S ribosomal RNA gene, partial sequence  
CTCCATCCAGTGGTGATCGGGTGAGTCCCACGTTTGTAACTGCCCAGAAGCGGGGGACAACAT  
TTGGAAACAGACGCTAATACCGCATAACAGCGCCGCTCGCATGAACAACGCTCATAACGATGGCT  
TCTCGCTATCACTTCTGGATGGACCTGCGGTGCATTAACCTGTTGGCGCGGTAAAGGCCTACCA  
AGGCCACGATGCATATCCGTGTTTAAAGACCGCTCGGCCACTATGTGATTGACACACATCCCAT  
ACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGCAAGCCTGATGGAGCAACACC  
GCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAGCTCTGTTGTTAAAGAAGAACACGTATGAGAGT
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AACTGTTTCATACGTTGACGGTATTTAACCAGAAAGTCACGGCTAACTACGTGCCAGCAGCCGCG
GTAATACGTAGGTGGCAAGCGTTATCCGGATTTATTGGGCGTAAAGAGAGTGCAGGCGGTTTTTC
TAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCATCGGAACTGGATAACTTTGAGT
GCAGAAGAGGGTAGTGGAACCTCCATGTGTAGCGGTGGAATGCGTAGATATATGGAAGAACACCA
GTGGCGAAGGCGGCTACCTGGTCTGCAACTGACGCTGAGACTCGAAAGCATGGGTAGCGAACAG
GATTAGATACCCCTGGTAGTCCATGCCGTAACGATGAGTGCTAGGTGTTGGAGGGTTTCCGCC
TTCAGTGCCGGAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTC
AAAGGAATTGACGGGGGCCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCTACGCGAAG
AACCTTACCAGGTCTTGACATCTTGCGCCAACCCTAGAGATAGGGCGTTTCTTTCGGGAACGCA
ATGACAGGTGGTGCATGGTTCGTGTCAGCTCGTGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTTGTACTAGTTGCCAGCATTAAAGTTGGGCACTCTAGTGAGACTGCCGGTGACA
AACCGGAGGAAGGTGGGGACGACGTCAGATCATCATGCCCTTATGACCTGGGCTACACACGTG
CTACAATGGACGGTACAACGAGTCGCGAACTCGCGAGGGCAAGCAAATCTCTTAAACCCTTCT
CAGTTCGGACTGCAGGCTGCAACTCGCCTGCACGAAGTCGGAATCGCTAGTAATCGCGGATCAG
CATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTACACCCATGAGAGTTTTGTT
ACACCCAAAGTTCGGTGGGGTAACT

16s rRNA sequence of *Lactobacillus brevis* (GeneBank Accession no. KU529283)

>gi|978128123|gb|KU529283.1| *Lactobacillus brevis* strain K38A
16S ribosomal RNA gene, partial sequence
ACGTGCTTGCCTGATTTCAACAATGAAGCGAGTGGCGAACTGGTGAAGTAACACGTGGGGAATC
TGCCCAGAAGCAGGGGATAACACTTGGAAACAGGTGCTAATACCGTATAACAACAAAATCCGCA
TGGATTTTGTGAAAGGTGGCTTCGGCTATCACTTCTGGATGATCCCGCGGCGTATTAGTTAG
TTGGTGAAGTAAAGGCCACCAAGACGATGATACGTAGCCGACCTGAGAGGGTAATCGGCCACA
TTGGGACTGAGACACGGCCAACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGAC
GAAAGTCTGATGGAGCAATGCCGCGTGAAGAAAGGGTTTCGGCTCGTAAACTCTGTTGTT
AAAGAAGAACACCTTTGAGAGTAAGTGTCAAGGGTTGACGGTATTTAACCAGAAAGCCACGGC
TAACTACGTGCCAGCAGCCGCGTAATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGT
AAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTTAACCGGAGAAGTGCA
TCGGAAACTGGGAGACTTGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGGAATGC
GTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTGTCTAGTCTGTAAGTACGCTGAGGCT
CGAAAGCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAACGATGAGTGCT
AAGTGTGGAGGGTTTCCGCCCTTCAGTGTGACGCTAACGCATTAAGCACTCCGCCTGGGGAG
TACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCCGCACAAGCGGTGGAGCATGTGG
TTTAATTCGAAGCTACGCGAAGAACCCTTACCAGGTCTTGACATCTTCTGCCAATCTTAGAGATA
AGACGTTCCCTTCGGGGACAGAATGACAGGTGGTGCATGGTTGTGTCGTCAGCTCGTGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAGCATTAGTTGGGCAC
TCTGGTGAAGTACCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCT
TATGACCTGGGCTACACACGTGCTACAATGGACGGTACAACGAGTCGCGAAGTCGTGAGGCTAA
GCTAATCTCTTAAAGCCGTTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTTGGA
ATCGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCC
CGTACACCCATGAGAGTTTTGTAACACCCAAAGCCGG

Table.1 List of selected isolates with their phenotypical characterization

S.No.	Fermented Food Sample	Traditional name of collected fermented food sample(s)	Place of purchased fermented food sample(s)	Isolate Code	Morphological characteristics	Gram's Reaction	Microscopic Examination
1	Fermented Soybean	<i>Tungrymbai</i>	Nongkrem, Shillong	RD10	Small, oval, white	+ve	Bacilli
2	Fermented Soybean	<i>Tungrymbai</i>	Tikrikela, Garo Hills	K38A	Pinpoint, Transparent	+ve	Short thick rods
3	Fermented Soybean	<i>Tungrymbai</i>	Asanang, Garo Hills	K20	Pinpoint, transparent	+ve	Bacilli

Table.2 Oligonucleotide sequences for PCR amplification

Primers	
27 F	5' TACGGYTACCTTGTTACGACTT 3'
1492 R	5' AGAGTTTGATCMTGGCTCAG 3'

Table.3 Biochemical tests of screened isolates through API 50CH kit

	API 50 CH	RD10	K38A	K20
0	Control	-	-	-
1	Glycerol	-	-	-
2	Erythritol	-	-	-
3	D-Arabinose	-	-	-
4	L-Arabinose	+	+	-
5	D-Ribose	+	+	-
6	D-Xylose	+	-	-
7	L-Xylose	-	-	-
8	D-Adonitol	-	-	-
9	Methylβ-D-Xylopyranoside	-	-	-
10	D-Galactose	+	+	-
11	D-Glucose	-	+	+
12	D-Fructose	+	+	+
13	D-Mannose	-	+	-
14	L-Sorbose	-	-	-
15	L-Rhomnose	-	-	-
16	Dulcitol	-	-	-
17	Inositol	-	-	-
18	D-Mannitol	-	+	-

19	D-Sorbitol	-	-	-
20	Methyl α -D-Mannopyranoside	-	+	-
21	Methyl α -D-Glucopyranoside	-	-	-
22	N-Acetyl Glucosamine	+	+	+
23	Amygdalin	-	+	-
24	Arbutin	-	+	-
25	Esculin Ferric citrate	+	+	+
26	Salicin	-	+	-
27	D-Celiobiose	-	+	-
28	D-Maltose	+	+	-
29	D-Lactose (bovine origin)	-	+	-
30	D-Melibiose	+	+	-
31	D-Saccharose(Sucrose)	-	+	+
32	D-Trehalose	-	+	+
33	Inulin	-	-	-
34	D-Melezitose	-	-	+
35	D-Raffinose	-	+	-
36	Amidon (starch)	-	-	-
37	Glycogen	-	-	-
38	Xylitol	-	-	-
39	Gentiobiose	-	+	-
40	D-Turanose	-	-	-
41	D-Lyxose	-	-	-
42	D-Tagatose	-	-	-
43	D-Fucose	-	-	-
44	L-Fucose	-	-	-
45	D-Arbitol	-	-	-
46	L-Arbitol	-	-	-
47	Potassium Gluconate	+	-	-
48	Potassium 2-KetoGluconate	+	-	-
49	Potassium 5-ketoGluconate	+	-	-
	Catalase test	-ve	-ve	-ve
	Identified <i>Lactobacillus</i> spp.	<i>L.brevis</i>	<i>L. brevis</i>	<i>L.fermentum</i>

Table.4 NCBI GeneBank accession number of the identified *Lactobacillus* isolates

Isolates	Partially identified by BLAST	NCBI GeneBank accession no.
K38A	<i>Lactobacillus brevis</i>	KU529283
K20	<i>Lactobacillus fermentum</i>	KU644579
RD10	<i>Lactobacillus brevis</i>	KX572212

Fig.1 Gram staining of the selected isolates

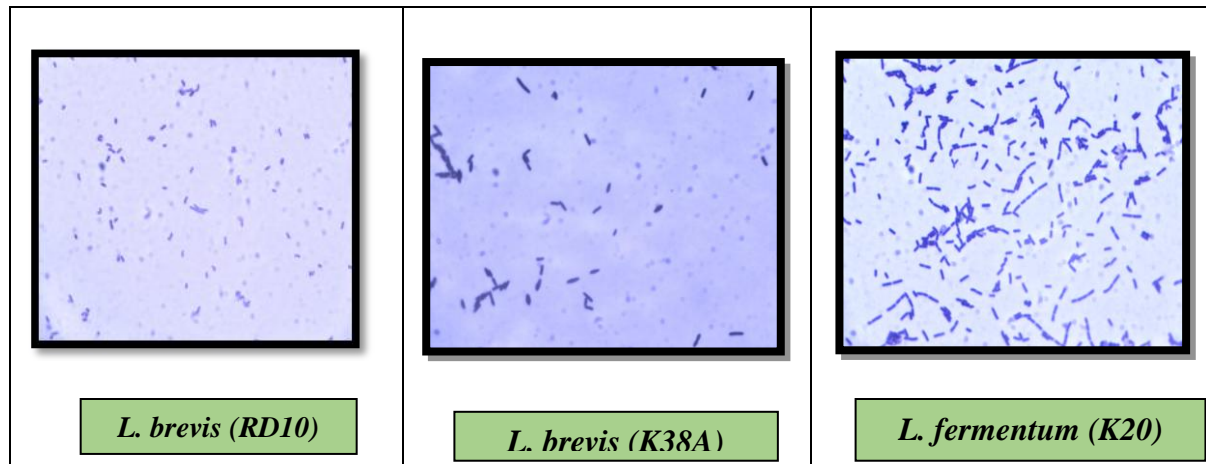


Fig.2 Biochemical analysis of the selected strain

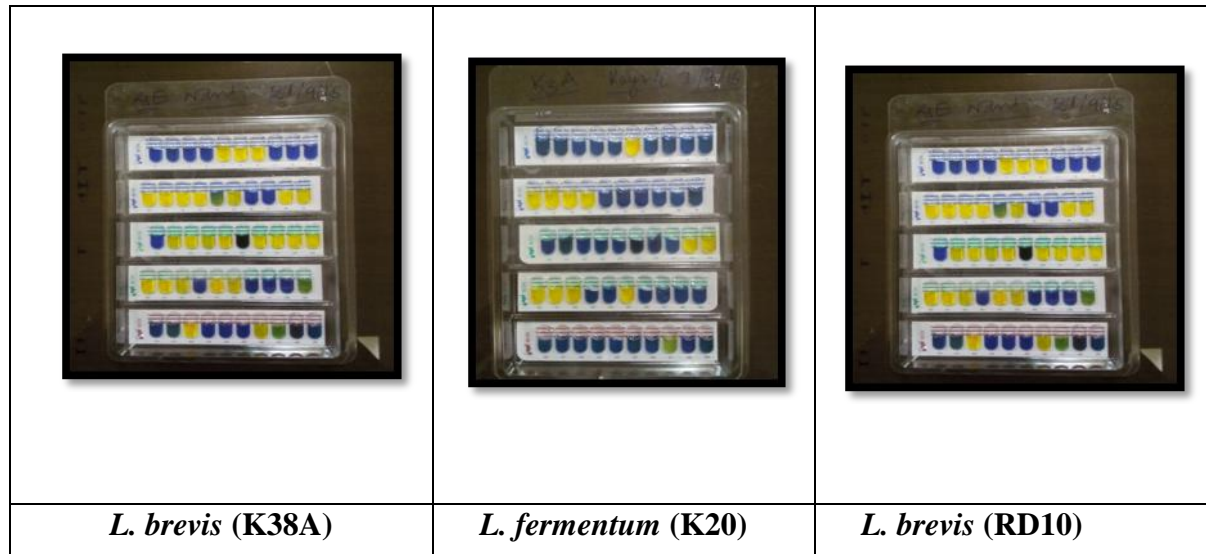
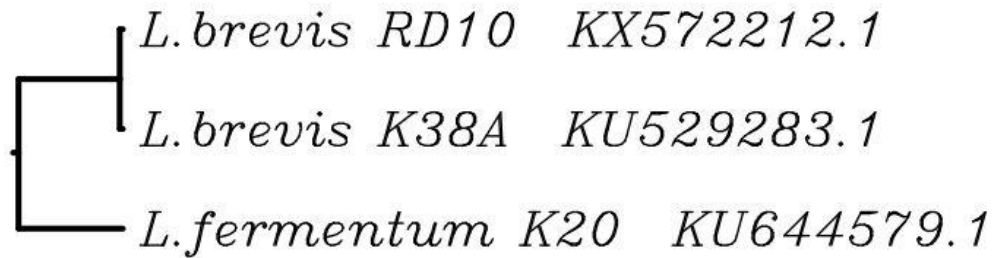


Fig.3 Rooted phylogenetic tree (UPGMA) for the strains of *Lactobacillus* from Tungrymbai



Phylogenetic analysis

To determine the closest known relatives of the partial 16S rDNA sequences obtained, nucleotide database searches were performed in NCBI GenBank and later the sequences were analysed by multiple sequence alignment tools using the DNA alignment program MAFFT v6.864 to signify the evolutionary relatedness between the strains by UPGMA (Unweighted Pair Group Method with Arithmetic Mean)

Hence, from the phylogram (Figure 3), it can be stated that the two isolates of *Lactobacillus brevis* are closely related due to the sequence similarity match as well the nodal distance. The other distantly related isolate is of *Lactobacillus fermentum* that connects the branch of the two closely related *Lactobacillus brevis* strains. Each node with descendants represents the inferred most recent common ancestor of the descendants which in this case is *Lactobacillus*.

The present study concluded that *Lactobacillus* spp. were predominant in microflora of *Tungrymbai*. The tentative phenotypic identification of the three isolates (RD10, K38A, and K20) were in good concordance with those by the genetic identification which derived that all the strains belonged to *Lactobacillus* spp. Further 16S rDNA sequence analysis of *Lactobacillus* strains confirmed the strains as *Lactobacillus*

brevis (K38A and RD10) and *Lactobacillus fermentum* (K20). The evolutionary relatedness was checked by connecting them through a phylogram. Further, the strains can be checked for their specific probiotic attributes that could be exploited for the development of value added fermented foods.

Acknowledgement

The authors are grateful to the Department of Biotechnology (DBT), Ministry of Science and Technology, New Delhi, India for the financial assistance under Twining Project - 2013 in collaboration with Anand Agricultural University, Anand, Gujarat and North-Eastern Hill University, Tura Campus, Meghalaya.

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How to cite this article:

Birendra Kumar Mishra, Subrota Hati, Sujit Das and Keyur Patel. 2017. Identification and Characterization of *Lactobacillus* isolates from fermented soya food "Tungrymbai", Meghalaya, India. *Int.J.Curr.Microbiol.App.Sci*. 6(2): 1103-1112.
doi: <http://dx.doi.org/10.20546/ijcmas.2017.602.124>