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Full Length Research Paper

Identification of *Lactobacillus pobuzihii* from *tungtap*: A traditionally fermented fish food, and analysis of its bacteriocinogenic potential

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Lactic acid bacteria (LAB) were selectively isolated from t*ungtap*, a traditionally fermented fish food product. Five such bacteria with bacteriocinogenic potential were characterized by polyphasic taxonomic approach. The phylogenetic analyses of 16S rRNA gene sequences showed their relatedness to *Lactobacillus pobuzihii* (≥99.4% similarity), while biochemical and physiological characteristics revealed significant differences among the isolates. Bacteriocinogenic activity assay against selected bacterial strains: *Salmonella typhi* MTCC 733*, Bacillus cereus* MTCC 430, *Klebsiella pneumoniae* MTCC 109, *Escherichia coli* MTCC 118 and *Bacillus licheniformis* MTCC 429 were performed with crude extracts using ethyl acetate as solvent, as well as by solvent and cell free supernatants. The growth inhibition zones were measured after incubation and compared with antibiotics like ampicillin, gentamicin, vancomycin, chloramphenicol and tetracycline. The culture extract of *L. pobuzihii* showed significant bacteriocinogenic activity against the tested strains.

Key words: Fermented, *tungtap*, *Lactobacillus pobuzihii,* crude extracts, bacteriocinogenic activity.

INTRODUCTION

Fermentation has long been known and applied by humans for making different foodstuffs and bacteria are known to be associated with such fermentation (Salminen and Wright, 1998). It has been known that home based fermented food products have higher species composition of lactic acid bacteria when compared with those of the trade products (Tserovska et al., 2002), and the wild strains from biotechnological viewpoint are prospective producers of bacteriocin (Leisner et al., 1999; Vaughan et al., 2001) and probiotics (Holzapfel et al., 2001; Kitazawa et al., 2001).

Tungtap is a traditionally fermented fish food product prepared from *Puntius* sp. by the Khasi and Jaintia tribes of North- East India. This fermented food is prepared by mixing dried fish with fresh fish fats, packed and stored in air-tight earthen pots, and incubated for 2 to 3 months. Some reports on microbial composition of *tungtap* are available (Thapa, 2004; Nair and Surendran, 2005), but our study intended to isolate selectively only the lactic acid bacteria (LAB) that has bacteriocinogenic potential. LABs are reported to be active in performing fish fermentation (Tanasupawat et al., 1998) and are widely distributed in nature with probiotic importance (Tserovska et al., 2002). They are Gram-positive, usually non-motile and non-sporulating bacteria, and produce lactic acid as a major or sole product of fermentative metabolism.

In this study, we identified five *Lactobacillus pobuzihii* owing to their high antibacterial activity against certain indicator bacterial strains which are often the source of food borne diseases or food poisoning due to contamination. *L. pobuzihii* has been identified previously from pobuzihi fermented seeds (Chen et al., 2010), but it's identification from *tungtap* is reported for the first time.

MATERIALS AND METHODS

Isolation of lactic acid bacteria (LAB)

Fresh fermented fish samples (*tungtap*) were collected in sterile

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container from retail markets in and around Shillong in Meghalaya, and were transferred to the laboratory in ice box. The samples of fish (skin with muscle) were homogenized in a blender using sterile saline water (0.85% w/v NaCl) and diluted inocula were plated on De Man Rogosa and Sharpe (MRS) agar (De Man et al., 1960). The plates were incubated at 37°C for 48 to 72 h and colonies were randomly picked up from the plates. Purity of the cultures was confirmed by streak plate method using nutrient agar medium and the cultures were preserved using 15% glycerol.

Phylogenetic analyses

Genomic DNA was extracted from the selected LAB. 16S rRNA gene sequences were amplified by PCR using two general bacterial 16S rRNA gene primers 27F/20 5′-AGAGTTTGATCCTGGCTGAG-3′ and 1541/20 5′-AAGGAGGTGATCCAGCCGCA-3′ respectively (Weisburg et al., 1991) in a PCR Gene Amp 9700 (Applied Biosystems, California, USA) with 25 µl volume reaction mixture containing 0.6 U *Taq* polymerase (Fermentas, Germany) with the corresponding buffer, 1.5 mmol/L $MgCl_2$, 0.125 mmol/L of each of dATP, dTTP, dGTP and dCTP, 10 pmol of each primer and 50 ng DNA. PCR was carried out at 94°C denaturation temperature for 5 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min and a final step at 72°C for 5 min. Approximately 1500 nucleotides were amplified using PCR. DNA template replaced with sterile water was always used as negative control. Amplified products were purified using QIAquick Gel Extraction Spin Kit (QIAGEN, Germany).

The purified 16S rRNA gene products were used as templates and were bi-directionally sequenced using the forward, reverse and internal primers corresponding to *Escherichia coli* positions 357F and 1100R. Sequencing reaction was performed with a 20 µl reaction mixture containing 5.5 µl purified PCR product (~50 ng of DNA), each primer at a concentration of 5 µM, 4 µl of terminator ready reaction mixture (consisting of Big Dye 3.1, Big Dye Buffer) and sterile water. Post reaction cleanup and re-suspension was performed for removal of unincorporated dye terminators from the sequencing reaction using 125 mM EDTA, 3 M sodium acetate and ethanol (70%). Sequencing was performed using Genetic Analyzer ABI 3130XL (Applied Biosystems, California, USA).

Sequence similarities were determined by the BLAST (Altschul et al., 1997) program against the database of type strains with validly published prokaryotic names at the EzTaxon server (http://www.eztaxon.org/; Chun et al., 2007). Molecular Evolutionary Genetics Analysis software (MEGA version 4) (Tamura et. al., 2007) was used for the phylogenetic analyses. The sequences of identified phylogenetic neighbors were aligned with the sequences of representative strains (Heilig et al.,2002), using ClustalW inbuilt with MEGA 4. *Lactobacillus plantarum* NBRC 15891T was used as the outgroup organism. Neighbor-joining method was employed to construct the phylogenetic tree with 1000 bootstrap replications to assess nodal support in the tree.

Nucleotide sequence accession numbers

Partial 16S rRNA gene sequences of all the five strains were submitted to NCBI GenBank. Accession numbers obtained are: *Lactobacillus* sp. TTp4, HQ141620; *Lactobacillus* sp. TTp6, HQ141621; *Lactobacillus* sp. TTp12, HQ141622; *Lactobacillus* sp. TTp13, HQ141623; *Lactobacillus* sp. TTp14, HQ141624.

Physiological and biochemical characteristics

Biochemical profiling included Gram staining, catalase, cytochrome oxidase, Hugh Leifson test, methyl red and Voges-Proskauer test, nitrate reduction, citrate test and indole production. Physiological study comprising of growth at 15°C and 45°C for 1 week and motility was also checked. API 50CHL sugar fermentation kits (Biomerieux, France) were used to study acid production from 50 carbohydrates. Presence (1) or absence (0) of each of the phenotypic trait of the representative strains as well as the type strain *Lactobacillus fermentum* MTCC 903 were constructed as a binary matrix and imported into NTSYS-pc software version 2.02 k for cluster analysis (Rohlf, 1998). Phenotypic similarity among all individuals was calculated according to simple matching coefficient using the SIMQUAL similarity program (Everitt and Dunn, 1992). The similarity coefficients were used to perform cluster analysis with the SAHN program employing the unweighted pair group method with simple arithmetic average (UPGMA).

Assay of antibacterial activities

Isolated colonies of LAB were screened for antibacterial-producing activity using the spot method as described by Spelhaug and Harlander (1989). An overnight culture of the test organism grown in MRS broth supplemented with 2.5% yeast extract (MRSY) was diluted 10-fold in 10 mmol/L Tris HCl (pH 7.0), and 0.2 ml of the aliquot was spotted onto MRS agar. Plates were incubated for approximately 48 h until growth was evident, and then overlaid with 5 ml trypticase soft agar (0.7% agar) seeded with 0.1 ml of an overnight culture of indicator bacterial strains. Plates were incubated for an additional 18 h and checked for clear zones around spots of the putative producers.

Bacteriocinogenic activity of the strains

Bacteriocinogenic activity assay was performed using two methods. First, with crude extracts using ethyl acetate as solvent, and secondly using solvent and cell free culture supernatant of the isolates obtained from 5 days old cultures (Amin et al., 2009) in MRS broth media supplemented with 20 mM of glycerol. For extracting supernatant using solvent, it was diluted 1:10 (ethyl acetate: culture broth) and agitated for 24 h. The mixture got separated into two phases from where the white fractionated supernatant was extracted using separating funnel. Ethyl acetate solvent was used because ethyl acetate extracts are reported to be more effective than methanol and ethanol extracts (Patra et al., 2009). In the second method using solvent and cell free supernatant (CFS), the broth culture media of the isolates were filtered using Whatman filter paper and exposed under UV (Ogunbanwo et al., 2003) for 30 min. The filtrate was centrifuged at 10000 RCF for 10 min. Both supernatants (solvent and solvent free) were concentrated in a rotary evaporator at 37°C in order to exclude solvent and other volatile substances.

The weight of the concentrated compounds was then determined (Amin et al., 2009) and re-suspended in 0.05 M potassium phosphate buffer (pH 7.0) and kept in cryovials in refrigerator (Patra et al., 2008) at 4°C until used. The sensitivity of the indicator bacterial strains was tested in both the solvent extracted crude extracts and CFS after 24 h incubation at 37°C using modified E-test (Amin and Kapadnis, 2005) as well as agar well diffusion assay (Schillinger and Lucke, 1989) in Mueller Hinton (MH) agar medium (Himedia, India). Agar wells of 6 mm diameter were made in MH agar plates using gel puncher. 150 µl of the culture supernatants was introduced in two wells, whereas 20 µl standard antibiotics (1 mg/ml) diluted with distilled water was added to the third well. Selected bacterial strains; *Salmonella typhi* MTCC 733, *Bacillus cereus* MTCC 430, *Klebsiella pneumoniae* MTCC 109, *Bacillus licheniformis* MTCC 429 and *E. coli* MTCC 118 were assayed as indicator strains for bacteriocinogenic activity. The zone of inhibition developed on the plates by culture supernatants were compared with antibiotics like ampicillin, gentamicin,

chloramphenicol, tetracycline and vancomycin.

Sensitivity of bacteriocins to heat, pH treatments and hydrolytic enzymes

Percentage of residual antimicrobial activity of solvent and cell free supernatants of the isolated cultures was assayed by exposing to heat treatments at 80°C for 20 min, 100°C for 20 min and 120°C for 20 min at neutral pH (6.5). In order to determine the effect of pH on unpurified bacteriocin, the extracts were treated to various pH values in the range of 5 to 8. The pH-treated crude extracts samples were incubated at 37°C for 20 min and then neutralized to pH 6.5 by means of 1 M NaOH and 1 M HCl and used for testing of bacteriocinogenic activity.

The following enzymes were also tested for their hydrolytic activity on the antibacterial compounds contained in the supernatants: proteinase K (2.6 U/mg), pronase E (22 U/mg), pepsin (16 U/mg) and α-amylase (15 U/mg). The assays were performed at a final concentration of 0.5 mg/ml and at pH 6.5, except for pepsin (pH 3.0). Samples with and without enzymes were held at 37°C for 6 h and the remaining activity was determined by agar well-diffusion assay as aforementioned using the selected indicator strains.

RESULTS

Identification of the isolates

Isolates were randomly picked up based on unique colony characteristics from MRS agar plates. After secondary screening of antagonistic activity, 30 Gram-positive and catalase negative isolates were selected for further studies. All of these 30 bacterial isolates were chosen for further molecular analysis. As many as ten (33%) of these isolates were found to be closely related to *L. pobuzihii* (≥98% maximum identity) when the BLAST program was performed against the nucleotide database of NCBI. Of these ten isolates, five representative isolates exhibiting superior antagonistic activity were selected for further analyses comprising phylogenetic, physiological, biochemical and bacteriocinogenic assay.

More than 1400 base pairs of 16S rRNA gene sequences of the five representative isolates were used to perform BLAST program against the database of type strains at EzTaxon server to identify the nearest phylogenetic neighbors. It produced nearest homology (>99.4% similarity) with *L. pobuzihii* E100301T (KCTC 13174, NBRC 103219). Consequently, sequences with high similarity scores were retrieved and phylogenetic tree was created using MEGA (version 4). Other members of the family Lactobacillaceae which displayed close sequence similarity were also included to study their relatedness with the representative isolates. Four major clusters were obtained. The isolates TTp4, TTp6, TTp12, TTp13 and TTp14 clustered together with *L. pobuzihii* with good bootstrap support (Figure 1).

Physiological and biochemical characteristics

The colony of five *L. pobuzihii* isolates and the type strain *L. fermentum* MTCC 903 were round and off white in colour. They were Gram-positive rods, fermentative, facultatively anaerobic, non-motile, catalase negative and did not possess endospore. None of the isolates were found to be fermenting xylose, melebiose, melizitose, aesculin, rhamnose and mannose. TTp4, TTp6 and TTp13 were found to ferment galactose, TTp6 and TTp12 were found to ferment salicin and TTp12 and TTp13 were found to ferment trehalose. The isolate TTp6 only could ferment raffinose. All of the isolates were found to produce acid homo-fermentatively from D-glucose, but did not produce gas from the same test sugar. None of the isolates showed positive test for nitrate utilization, urea, gelatin, casein, citrate and indole production, whereas *L. fermentum* MTCC 903 was found to be citrate positive (Table 1). When dendrogram was created using NTSYS-pc software against biochemical data, all of these five isolates clustered together, except the isolate TTp12 that clustered with *L. fermentum* (Figure 2).

Antibacterial activity

Putative antibacterial-producing LAB isolated from fermented fish *tungtap* was detected using the spot method assay on the basis of their ability to inhibit growth of the indicator bacterial strains. Five of these isolates which were closely related to *L. pobuzihii* were found to produce bacteriocin-like substances. All of these five inhibitor-producing isolates that were selected for further study on the basis of their relatively wide antibacterial spectrum consistently showed their activity with the culture supernatants.

Antibacterial activity of the culture extracts was checked for some selected bacteria which are opportunistic food borne bacterial pathogens (Mechai and Kirane , 2008). The culture extract of *L. pobuzihii* showed significant bacterio-cinogenic activity, although not as effective as some antibiotics (Figure 3). The zone of inhibition was higher in CFS compared to solvent extracted supernatants Figure 3C. The isolate TTp14 showed 7 mm inhibition zone in plate inoculated with 200 µl (1.308×10^7 CFU/ ml) of *B. cereus*. The isolate TTp13 showed an inhibition zone of 8 mm against *S. typhi.* CFS extracted from TTp6, TTp12, TTp13 and TTp14 showed significant zone of inhibition against *K. pneumoniae* and *E. coli* (Figure 3A to C). The zones of inhibition so generated from the extracts were compared with standard antibiotic (20 µg). Bacteriocins produced by few isolates remained stable during heat treatment up to 120°C for 20 min. The isolate TTp13 showed higher thermal stability and percent residual antibacterial activity was observed against *K. pneumoniae*. However, the antibacterial activity of the same temperature treated extracts for the same isolates was reduced to 60% against *E. coli*. On treatment with hydrolytic enzymes such as proteinase K, pronase E, pepsin and α-amylase, the extracts lost their broad spectrum inhibitory activity

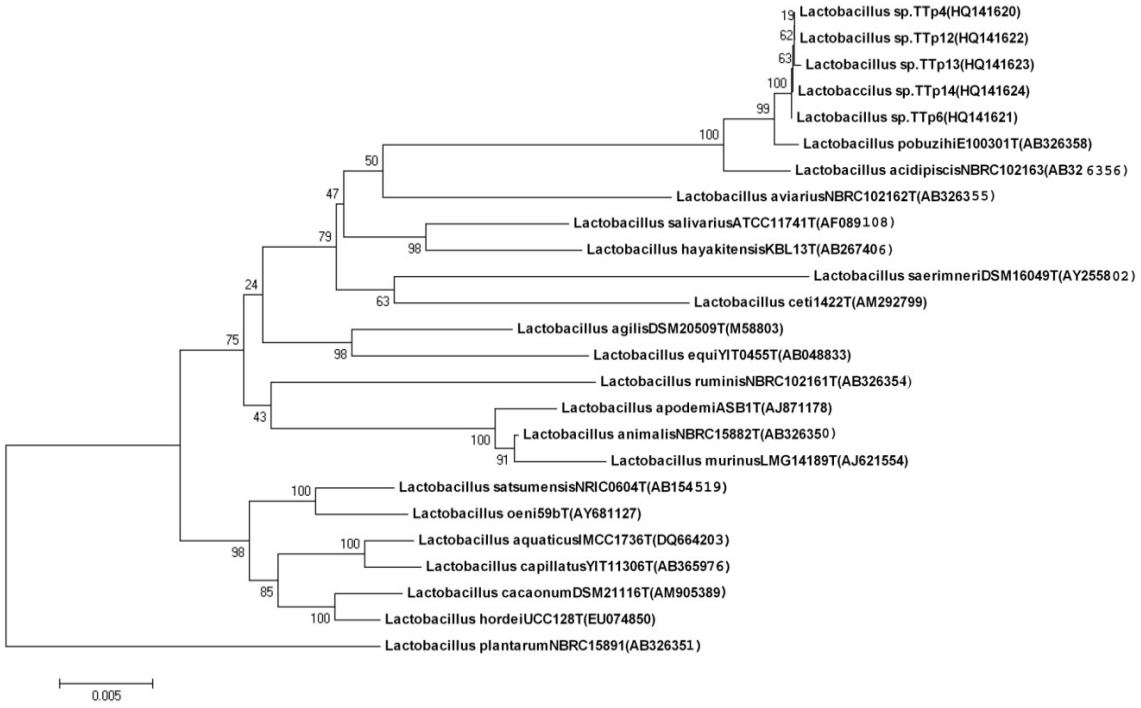

Figure 1. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequence comparisons showing the position of strain *Lactobacillus* sp. (TTp4, TTp6, TTp12, TTp13 and TTp14) and related *Lactobacilli* (bootstrap values are indicated at branch points based on 1000 replications). The sequence of *Lactobacillus plantarum* NBRC 15891T was used as an outgroup. GenBank accession numbers are given in parentheses

Table 1. Morphology and biochemical characteristics of the five bacterial Isolates and type strain *Lactobacillus fermentum* (MTCC 903).

Characteristic		TTp4	TTp6	TTp12	TTp13	TTp14	MTCC 903
Methyl Red Test		+	+	-	+	+	+
VP- test		+	-	-	-	-	-
Oxidase test		+	+	+	+	+	-
Citrate utilization test		-	-	-	-	-	+
Starch utilization test		-	-	+	+	-	-
Lipid hydrolysis		-	-	+	-	-	-
HL Test	Oxidative	-	-	+	+	-	-
	Fermentative	+	-	+	+	+	+
Sugar fermentation test	Cellobiose	-	+	+	-	-	-
	glucose	+	-	+	-	-	-
	Salicin	-	+	+	-	-	-
	Trehalose	-	+	+	+	-	-
	Mannose	+	+	+	+	-	-
	Raffinose	-	+	-	-	-	-
	Galactose	+	+	-	+	-	-
Growth in NaCl Solution (%)	4	+	+	+	+	+	+
	6	+	+	+	+	+	+
	10	+	+	+	+	+	+
	12	-	+	+	+	-	-

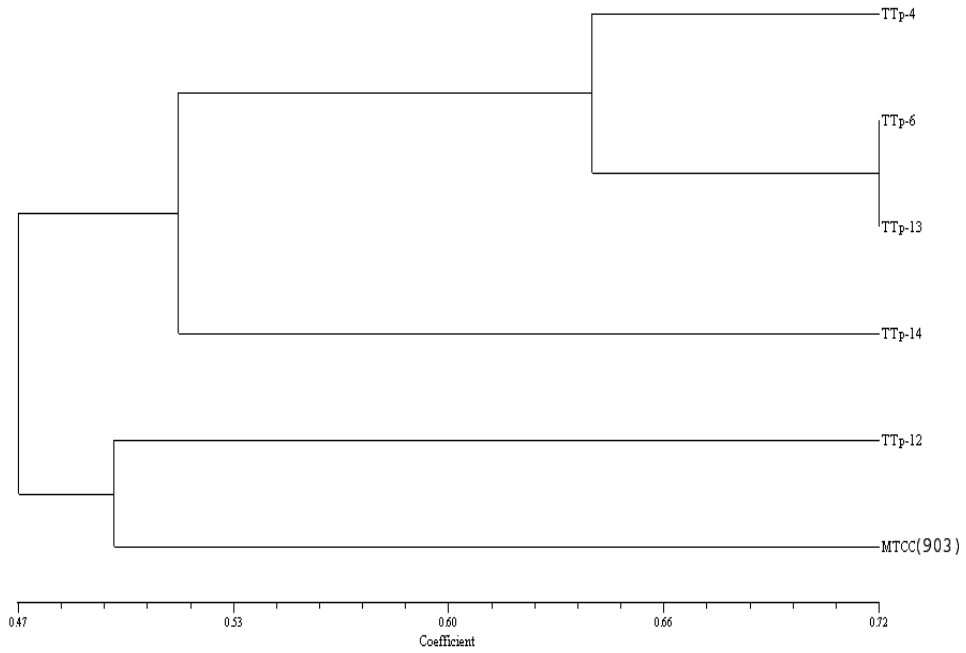

Figure 2. Phylogenetic dendrogram using UPGMA clustering analysis of the biochemical profile of the five *Lactobacillus* sp. TTp4, TTp6, TTp12, TTp13 and TTp14 isolates and reference strain *Lactobacillus fermentum* MTCC 903.

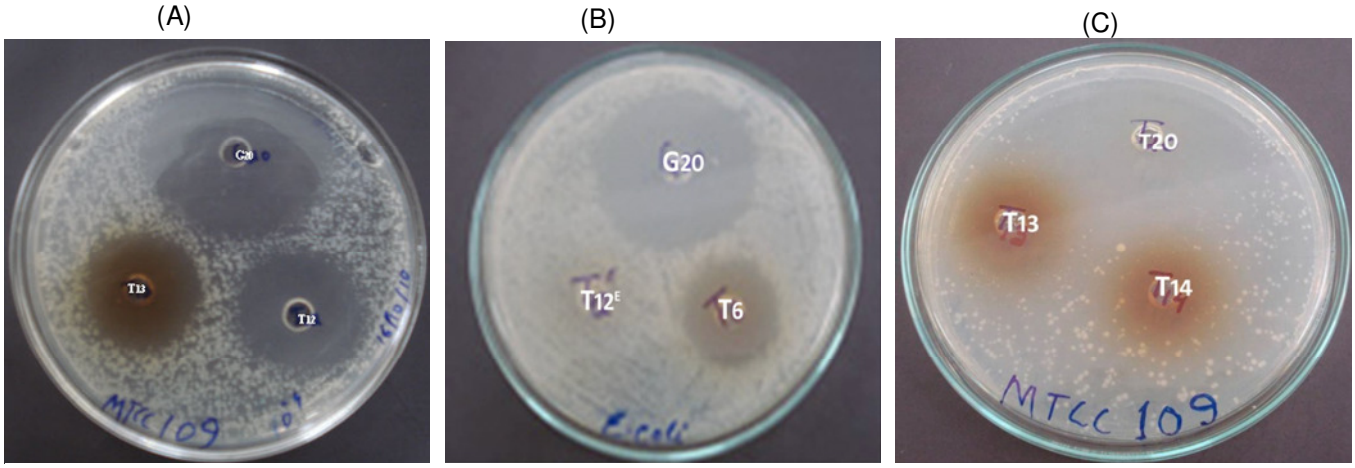

Figure 3. (A) Inhibition of *Klebsiella pneumoniae* MTCC 109 by cell free supernatants of the two producing isolated strains using agar well diffusion assay: *Lactobacillus pobuzihii* TTp13 (well T13), TTp12 (well T12), gentamicin (well G20). Inoculum used: 2.616×10^7 CFU. (B) Inhibition of *E. coli* by cell free supernatants (TTp6) and ethyl acetate solvent extracted supernatant (TTp12) of the two producing isolated strains using agar well diffusion assay: *Lactobacillus pobuzihii* TTp12 (well $T12^E$) and TTp6 (well T6). Inoculum used: 2.616×10^7 CFU. (C) Inhibition of *Klebsiella pneumoniae* MTCC 109 by antibacterial cell free supernatant of the two producing isolates of *Lactobacillus pobuzihii* using agar well diffusion assay: TTp13 (well T13), TTp14 (well T14) and tetracycline (well T20). Inoculum used: 2.616×10^7 CFU.

DISCUSSION

Isolation and screening of microorganisms from naturally occurring processes have always been the most powerful means for obtaining useful cultures for scientific and commercial purposes. Same applies for LAB, which plays an important role in a large number of traditional food fermentations (Vijai et al., 2004). Although in our study, the bacteria with antagonistic activity against indicator bacteria revealed the presence of diversity of LAB, we targeted a sub-group of *L. pobuzihii* for our investigation based on their superior bacteriocinogenic potential and

wide occurrence. The isolates TTp6, TTp12 and TTp13 were found to grow at 12% salt, whereas *L pobuzihii* isolated from pobuzihi seeds (Chen et al., 2010) were reported to grow only up to 10% salt. The high salt concentration created in the fish during traditional fermentation may have inhibited the growth of other unwanted bacteria and allowing only the salt tolerant to grow. CFS in this study was used as crude extracts, but is yet to be established whether the antibacterial activity comes from bacteriocin peptides.

The antimicrobial activity shown by the selected LAB isolates in this study could have acted as a barrier to inhibit food spoilage and/or growth of pathogenic microorganisms in the fermented fish food. Bacteriocin production besides other organic acids and hydrogen peroxide was reported in LAB from fermented foods, which are shown to inhibit growth of a broad range of Gram-positive as well as Gram-negative bacteria and prevent the growth of pathogenic bacteria (Jin et al., 1996). Gollop et al. (2004) reported LAB extracts isolated from silages showing antibacterial activity against various groups of bacteria. *Lactobacillus* spp. producing bacteriocin related compound have been reported in *L. salivarius* and *L. reuteri* (Cleusix et al., 2007). The inhibition zones developed in this study remained unchanged when the supernatant was treated with 1 M NaOH and adjusted to pH 6.5, indicating that the bacteriocin may be responsible for the growth inhibition of the indicator strains. Furthermore, when the crude extracts were treated with proteolytic enzymes such as trypsin, proteinase K, pronase E and α-amylase; there was no zone of inhibition as the supernatants lost their activity. This confirmed that the compounds are proteinaceous in nature, which was actually responsible for the growth inhibition of the indicator bacteria. To our knowledge, this is the first study on identification of *L. pobuzihii* and its antimicrobial activity from *tungtap* a traditionally fermented fish food of North-East India.

Conclusion

Preserved microorganisms from lesser-known fish product may contribute significant information on the unknown microbial gene pool as genetic resources. Considerable efforts have recently been focused on the understanding of the structure, the genetic organization and the mode of action of several bacteriocins. There has been a concomitant development in the description of new bacteriocins whose biochemical and genetic characterization could lead to the discovery of important elements for the elucidation of structure/function relationships in these substances. The interesting finding in this work was that the crude extract from the isolated LAB, *L. pobuzihii*, showed a positive significant activity against the selected indicator bacterial strains. The bacterial supernatants of these isolated strains, however, need to be evaluated for bioactive compounds like bacteriocins.

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