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Isolation and Molecular Characterization of *Bacillus* species from *Shidal* - A Fermented Fish Product of Assam

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ABSTRACT: *Bacillus spp. constitutes a diverse group of bacteria widely distributed in aquatic environment. In the present study, 20 nos. of Bacillus spp. were isolated from shidal, a fermented fish product of Assam. From these, two representative isolates were characterized by biochemical and molecular methods. The 16S rRNA sequencing and typing confirmed the isolates as Bacillus subtilis and B. amyloliquefaciens respectively.*

Key words: *Bacillus subtilis, Bacillus amyloliquefaciens, Fermented fish, Identification, Shidal*

INTRODUCTION

Traditional processing of fish such as fermentation, salting, drying and smoking are the principal methods of fish preservation in Southeast Asia (Cooke *et al.*, 1993). In North East India, fermentation is one of the oldest and most economical methods for producing and preserving food. In addition to preservation, fermented foods can also have the added benefits of enhancing flavour, increasing digestibility, improving nutritional value and pharmacological values.

Fermentation is brought about by microorganisms and enzymes present on the surface as well as gut of the fish. Each fermented product is associated with unique group of microflora which increases the level of protein, vitamins, essential amino acids and fatty acids (Jeyaram *et al.*, 2009).

Shidal is a salt-free, solid, semi-fermented product which is indigenous to north-eastern states of India and prepared from small sized fish mainly *Puntius* sp. (Muzaddadi *et al.*, 2003). It has several local names like *seedal, seepa, hidal* and *shidal* in Assam, Tripura, Arunachal Pradesh, Nagaland and *ngari* in Manipur. Shidal is one of such fermented fish products which is used both as flavouring and a source of protein. The characteristic taste and flavor of shidal is the reason for its acceptability and popularity among the tribals, Bengalis and other population of the above areas (Ahmed *et al.*, 2013).

Bacillus species have been isolated from nam-pla, a Thai fermented fish sauce (Saisithi *et al.*, 1966). It appears that not much information is available on the microbiological aspects of these traditionally processed fish products of Assam and other north-eastern states of India. The present study aimed at isolation and characterization of the predominant bacteria, involved in fermentation of shidal.

MATERIAL AND METHODS

Collection of Samples

Six samples of shidal were collected in aseptic conditions from a local dry fish market of Assam, packed tightly in pre-sterile polyethylene bags kept in an ice-box and air lifted to the laboratory. The aseptically collected semi-fermented fish shidal were minced and finely homogenized in pestle and mortar and used for microbiology.

Microbiological Analyses

In order to isolate the *Bacillus* species, 10 g sample was aseptically collected and macerated with 90 ml of 0.85% (w/v) sterile physiological saline (NSS) and

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homogenised in a stomacher lab-blender 400 (Seward, UK) for 1 min. Decimal dilution series were prepared in sterile diluents and 0.1 ml of each diluted suspension of the sample was spread onto MRS agar (HiMedia M641, India) plates, spread and incubated under anaerobic conditions in an Anaerobic jar (HiMedia, India) at 30°C for 48 days.

Isolation, Purification and Initial Characterization of Bacteria

Colonies were picked and purified by successive sub-culturing on MRS Agar plates to obtain pure colonies. The purified isolates were examined for colony morphology, Gram staining, spore formation and motility, as described by Cruickshank *et al.* (1975) and Cappuccino and Sherman (1999).

Identification of Bacteria

The isolates were identified up to genus level using morphological and biochemical attributes. The identification of the isolates to species level was confirmed by 16S rRNA gene sequencing and typing at the Institute of Microbial Technology (IMTECH), Chandigarh, India.

16S rRNA Gene Sequencing

Genomic DNA of the isolates selected for 16S rRNA gene sequencing was extracted using Wizard Genomic DNA Purification kit (Promega Corporation, Madison, WI, USA). One loopful of the selected isolate was inoculated into 9 ml of JCM containing 20% NaCl broth, and incubated at 35°C for 5 days. Bacterial cells at the late exponential phase were harvested by centrifugation at 12,000 rpm for 2 min at 4°C. The supernatant was discarded and the cell pellet was washed once with 240 µl of 50 mM EDTA (pH 8), then 30 µl of 10 mg ml^{-1} lysozyme was added. The mixture was incubated at 37°C for 30 min, followed by addition of 300 µl of nuclei lysis solution. Subsequently, samples were incubated at 80°C for 5 min and kept at room temperaturefor cooling. To the mixture, 1.5 µl of RNAse solution was added, mixed by inversion and incubated at 37°C for 30 min. One hundred microliter of protein precipitation solution was added and then mixed using a vortex device at high speed for 20 sec. After incubation at 0°C for 5 min, the mixture was centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was transferred into a new sterile microcentrifuge tube. Subsequently, 300 µl of isopropanol was added to the supernatant and centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was discarded and 600 µl of 70% ethanol added to the DNA pellet. The mixture was centrifuged at 12,000 rpm for 2 min at 4°C. After the supernatant was discarded, it was dried at 37 °C for 1 h. 50 microliter of TE buffer (10 mM Tris-HCl, pH 8 and 1 Mm EDTA) was added and kept overnight at 4°C to allow DNA to dissolve. The extracted DNA was detected using 0.8% agarose gel electrophoresis in TBE buffer (pH 8.3). Polymerase Chain Reaction (PCR) was performed in a Thermal Cycler with a primary heating step for 2 min at 94°C, followed by 30 cycles of denaturation for 60 sec at 94°C, annealing for 60 sec at 53°C and extension for 1.30 min at 72°C. Each 25 µl of reaction mixture contained 2 µl of genomic DNA, 14.25 µl of MilliQ water, 2.5 µl of 10x buffer (100 mM Tris-HCl, pH 8.3; 500 mM KCl), 1.5 µl of 25 mM $MgCl_2$, 2.5 µl of dNTPs mixture (dATP, dCTP, dGTP, dTTP at 10 mM each concentration), 1.0 µl of each primer (PO mod f, PC3 mod r, P3 mod f and PC5 r) (20.0 pmoles μl^{-1}), and 0.25 µl of *Taq* DNA polymerase. The PCR amplified products were examined by electrophoresis using 1% agarose gel containing ethidium bromide (0.5 µg ml^{-1}). The size of PCR products was compared with 1 Kb DNA ladder. The separated PCR products were observed under short wavelength UV light. Size of the PCR products was approximately 1,500 bp. Direct sequencing of 16S rRNA gene were performed using Primer and Terminator Ready Reaction kit version 2.0 (Perkin Elmer, Applied Biosystems, Inc., Foster, CA, USA) in combination with an automated sequencing system. The gene was amplified using Thermal Cycler. An estimated amount of 100 ng of DNA was used for each reaction together with 5 pmol of primer, 4 µl of ready reaction mix and DI water to attain a 10 µl final volume. The same primers were used as previous PCR amplification. Cycle-sequencing PCR and DNA precipitation with ethanol and sodium acetate were done following the manufacturer's protocol (Applied Bio-Systems, Foster, CA, USA). The precipited DNA was dried and dissolved in deionized water and then sequencing was performed.

RESULTS AND DISCUSSION

Out of 50 isolates picked from each sample. 20 isolates were identified as *Bacillus* species. Two distinct and predominant *Bacillus* isolates form the 20 *Bacillus* isolates were selected for further studies. Macroscopically, the colonies of the selected isolates were raised, milky white and brown in appearance. Brown and white colonies were sub-cultured in selective media in two halves. The bacterial isolates

which were milky white and brown were named as FPTB13 and FPTB16 respectively. Both the isolates were motile, spore forming, Gram +ve and catalase +ve rods and were identified as *Bacillus* species (Table 1).

Table 1
Morphological characteristics of isolates FPTB13 and FPTB16

Morphological characters	FPTB13	FPTB16
Grams staining	+	+
Shape	Coccobacillus	Bacillus
Spore formation	+	+
Motility	+	+

Based on the preliminary species level identification by biochemical profiling, the selected isolates FPTB13 and FPTB16 were classified as *Bacillus subtilis* and *Bacillus amyloliquefaciens*, respectively as per the description given by Priest *et al.* (1987). Biochemical characteristics of the isolates FPTB13 and FPTB16 are represented in Table 2.

Table 2
Biochemical characteristics of the isolates FPTB13 and FPTB16

Biochemical Test	*FPTB13*	*FPTB16*
Methyl red	+	+
Voges Proskauer	–	+
Oxidase	+	+
Catalase	+	+
Indole production	–	–
H_2S production	–	+
Citrate utilization	+	+
Starch hydrolysis	+	–
Casein hydrolysis	+	+
Nitrate reduction	+	+
Anaerobic growth	–	–

The identification of the isolates to species level was verified by 16S rRNA sequence homology as described in 16S rRNA gene sequencing method. The results of 16S rRNA identification of the isolates, FPTB13 and FPTB16 by 16S rRNA gene sequencing confirmed the isolates as *Bacillus subtilis* and *Bacillus amyloliquefaciens* respectively (BLAST Analysis data 1 and 2). Thapa *et al.* (2004) also identified endospore forming rods such as *Bacillus subtilis* from *Ngari* in Manipur. Sutyak *et al.* (2008) identified *Bacillus amyloliquefaciens,* a phylogenetically close relative of *Bacillus subtilis* through 16S rRNA analysis from a commercially available probiotic dairy beverage.

Bacillus subtilis (Isolate FPTB 13) was identified isolate was similar to NCBI database *Bacillus subtilis* strain HJ11 16S ribosomal RNA gene, partial sequence, whereas *Bacillus amyloliquefaciens* (Isolate FPTB16) was identified to be similar to NCBI database *Bacillus amyloliquefaciens* strain JX1 16S ribosomal RNA gene, partial sequence.

The nucleotide sequences of isolated two Bacillus species were directly submitted to the GenBank and after analysis of the sequences the following accession numbers were generated respectively:

GenBank accession number: KF319057
GenBank accession number: KF319058

CONCLUSION

Bacillus sp. has been identified as one of the predominant microbiological populations present in shidal which have wide scope for exploration as potent biopreservative in preparation of fermented fish products.

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