



Original Research Article

Isolation, Identification and Characterization of Protease Producing *Bacillus* spp. from Unexplored Ecosystems of Indo-Burma Biodiversity Hotspots

Onkar Nath Tiwari*, Kangjam Sarabati, Thiyam Bidyababy, Keithellakpam Ojit Singh, Oinam Avijeet Singh, Gunapati Oinam, Thingujam Indrama, Wangkhem Indira, Chungkham Silvia, Aribam Subhalaxmi, Romi Khangembam, Angom Thadoi and Longjam Miranda

Microbial Resources Division, Institute of Bioresources and Sustainable Development
(A National Institute of DBT, Govt. of India), Takyelpat, Imphal-795001, Manipur, India

*Corresponding author

ABSTRACT

A total of nineteen thousand eight hundred thirty four (19,834) purified bacterial isolates were encountered from Indian region falling under Indo-Burma biodiversity hot-spots and screened for protease activity in culture conditions. Twenty one (21) isolates were selected for detailed investigation on the basis of production of protease activity in culture conditions. Quantitative enzymatic assay revealed that amongst the 21 isolates, three (03) showed good amount of proteolytic activity i.e., 55.39 IU/ml, 56.02 IU/ml and 75.51 IU/ml at pH 4, 7 and 10 respectively which was at par with already commercialized isolates. These three isolates were characterized on the basis of their morphological, biochemical and molecular features and the isolates were identified as *Bacillus atrophaeus* (KJ562181), *Bacillus cereus* (KM010238) and *Bacillus coagulans* (KM010243). The ability of these isolates for high protease production could make them potential candidates for various commercial applications.

Keywords

Bacillus sp.,
Biochemical,
North-Eastern
India,
Characterization,
Protease activity,
16S rRNA

Introduction

Microorganisms are the most common sources of commercial enzymes due to their physiological and biochemical properties, facile culture conditions and ease of cell manipulation (Dias, 2010; Haki and Rakshit, 2003; Sumantha *et al.*, 2006). Proteases are one of the most important classes of enzymes, occupying a major share of 60% of total enzyme market (Verma *et al.*, 2011). These biocatalysts hydrolyze peptide bonds in proteins and hence are classified as

hydrolases and categorized in the subclass peptide hydrolases or peptidases (Ellaiah *et al.*, 2002). Proteolytic enzymes are ubiquitous in occurrence found in all living organisms and are essential for cell growth and differentiation. There is renewed interest in the study of proteolytic enzymes mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes also gained considerable attention in the industrial

community (Gupta *et al.*, 2005). Commercial proteases are mostly produced from various bacteria and it was reported that 35% of the total microbial enzymes used in detergent industry are the proteases from bacterial sources (Ferrero *et al.*, 1996). Culture conditions play significant role on growth and production of protease by bacteria (Shalinisen and Satyanarayana, 1993; Shumi *et al.*, 2004; Lee *et al.*, 1992). Based on their acid-base behaviour, proteases are classified into three groups i.e. acid, neutral and alkaline proteases. The majority of commercial alkaline proteases are produced by bacteria, especially *Bacillus* sp. (Denizci *et al.*, 2004). Several *Bacillus* species involved in protease production are *B. cereus*, *B. sterothermophilus*, *B. mojavensis*, *B. megaterium* and *B. subtilis* (Ammar *et al.*, 1991; Sookkheo *et al.*, 2000).

Identification and characterization of microbial proteases are pre-requisite for understanding their role in pathogenesis. Proteases are also useful and important components in biopharmaceutical products such as contact-lens enzyme cleaners and enzymatic debriders (Gouda, 2006).

The enzymatic yield obtained from fermentation, cost of their production and downstream processing cost determines the final cost of the enzyme produced (Gupta *et al.*, 2002). Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology (Kannan and Vincent, 2011). Currently, enzymes have attracted the world attention due to their wide range of industrial applications in many fields, including organic synthesis, clinical analysis, pharmaceuticals, detergents, food production and fermentation. Protease performs numerous varieties of activities in detergent, food, pharmaceutical, leather, laundry, food processing etc. These enzymes are widely used in dairy industry as milk

clotting agent and meat tenderizing agent in food industry, reduction of tissue inflammation application (Djamel *et al.*, 2009). The present study is to investigate the highest protease producing strains and their identification for commercial application.

Materials and Methods

Screening of protease producing microbes

A total of nineteen thousand eight hundred thirty four (19,834) bacterial isolates were collected from North-Eastern region of India (87°32'E-97°52'E longitude and 21°34'N-29°50'N latitude), belonging to the Indo-Burma biodiversity hot-spots and screened for protease activity. Out of the above screened isolates, twenty one (21) were streaked onto nutrient agar (HiMedia, India) medium and incubated at 37°C in BOD incubator (ORBITEK BOD350L, Scigenics Biotech, India) for 24 h for optimum growth and three best performed strains were selected for detailed investigation. Cultures were grown on nutrient agar plates and pre-screened by inoculation in skim milk agar (HiMedia, India) and kept for incubation at 37°C for 72 h, those which showed positive results in skim milk agar were selected for final screening and thorough investigation. These positive cultures were inoculated in skim milk agar medium of different pH (4.0, 7.0 and 10.0) and incubated at 37°C for 72 h. The clear zones of hydrolysis over the next 72 h were taken as evidence of proteolytic activity qualitatively.

Inoculum preparation for enzymatic assay

A loopful of culture was inoculated into 10 ml of Luria Bertani broth medium (HiMedia, India) and incubated at 37°C for 24 h. After this, 100 µl of the bacterial culture was inoculated to 20 ml of production medium (production medium

composed of glucose-0.5 gm, peptone-0.75 gm, MgSO₄-0.5 gm, KH₂PO₄-0.5 gm, FeSO₄-0.01 gm in 500 ml distilled water) and kept in a refrigerated incubator shaker (C24KC, New Brunswick Scientific Classic Series, NJ, USA) at 140 rpm for 72 h at 37°C. At the end of the fermentation process, the fermented broth was centrifuged at 10,000 rpm in a centrifuge (5430R, Eppendorf, Germany) for 10 min and the supernatant was separated and used as crude enzyme source for the assay of protease production.

Protease enzyme assay

Protease activity was determined by caseinolytic method (Tunga *et al.*, 2003). In this assay, 2 ml of 2% casein in 0.65 ml of respective buffer (acidic, neutral and alkaline phosphate buffers) was used. Casein solution was incubated with 0.5 ml of properly diluted enzyme at 37°C for 30 min. After 30 min, 200 µl of NaCl was added and then the reaction was terminated by addition of 5 ml of 5% TCA. The solution mixture was centrifuged at 10,000 rpm for 10 min. After centrifugation, 0.1 ml of the sample was taken and the volume was made up to 1 ml with distilled water. And 5 ml of solution-C (solution-A: In 50 ml distilled water, 0.2 gm NaOH was dissolved and 1 gm of Na₂CO₃ was added, solution-B: 0.5% CuSO₄ in 1% sodium potassium tartarate, solution-C: mixed 50 ml of solution-A + 1 ml of solution-B) was added and incubated at room temperature for 10 min. These were preceded by the addition of 0.5 ml Folin-Ciocalteu's reagent and incubated in dark room condition for 30 min. The colour developed was read at 660 nm against a reagent blank prepared in the same manner. One unit of proteolytic enzyme activity was defined as the amount of enzyme that liberated 1 µg tyrosine/ml/min from casein under specified assay conditions.

Biochemical tests

The protease producing strains with highest diameter of proteolytic zone were characterized biochemically and morphologically. Malonate, Vogues-Proskauer, Citrate, ONPG, Nitrate Reduction, Catalase, Arginine, Sucrose, Mannitol, Glucose, Arabinose, Trehalose were the biochemical test performed and gram's staining was performed using HiBacillusTM identification kit.

Genomic DNA extraction and PCR amplification

Genomic DNA was isolated phenol:chloroform isolation method (Newman *et al.*, 1990). 16S rRNA sequence was amplified from genomic DNA using universal primer (Integrated DNA Technology, India) 536F 5'-GTGCCAGCAGCCCGGGTRATA-3' and 1488R 5'-CGGTTACCTTGTTACGACTTCACC-3' (Nubel *et al.*, 1997). For the polymerase chain reaction (PCR), a total of 50 µl of PCR reaction mixture was prepared having 5 µl of 1X *Taq* buffer, 5 µl of 200 µM of each deoxynucleotide, 1.5 µl of 0.3 µM of each forward and reverse primer, 0.25 µl of 5U *Taq* DNA polymerase and 2 µl of genomic DNA extract. Amplification of DNA for 50 µL reaction was carried out under the following condition. The PCR conditions were set for 28 cycles with initial denaturation at 95°C for 5 min then final denaturation of 95°C for 1 min, annealing at 55°C for 1 min and final extension at 72°C for 2 min using Thermal cycler (Mastercycler gradient, Eppendorf, Germany). The PCR product was detected with standard agarose gel electrophoresis (Elchrom Scientific GEPS 200/2000, Switzerland) and quantification of PCR product was done with Biospectrometer (Eppendorf, Germany). Sequencing of the

quantified 16S rRNA PCR product was done at National Centre for Cell Science (NCCS), Pune, India. The sequences were analyzed using the gapped BLASTn analysis (<http://www.ncbi.nlm.nih.gov>) search algorithm for the correct identification of the bacteria and the sequences obtained in this study were deposited to the GenBank database.

Phylogenetic analysis of bacterial strain

The 16S rRNA sequences of all bacterial strains were aligned with reference sequences showing sequence homology from the NCBI database using the multiple sequence alignment program of MEGA 4.0 (Tamura *et al.*, 2007). Phylogenetic analysis of gene sequence data were conducted using the neighbor-joining (NJ) method (Saitou and Nei, 1987) by Kimura 2-parameter. The stability of trees obtained from the above cluster analyses was assessed by using BOOTSTRAP program in sets of 1000 replicates.

Results and Discussion

Protease producing microorganisms

Three (03) bacterial strains taken for the study showed varying proteolytic activities at different pH 4.0, 7.0 and 10.0 (Table 1). Screening of protease producing microorganism usually involves growth on the medium that contain protein as a selective substrate and in the present study skim milk was used as the substrate. Following inoculation and incubation of the agar plate, organisms secreted proteases exhibited a zone of proteolysis which was demonstrated by clear area surrounding the microorganism's growth. The strains which produced protease with 20-45 mm diameter of proteolytic zone were characterized and further processed for detailed studies (Fig. 1).

Three cultures showed highest proteolytic activity by distinctly forming clear zone around the colonies in skim milk agar plate of different pH 4.0 (25 mm), 7.0 (39 mm) and 10.0 (45 mm) respectively (Table 1). The zone formation around the bacterial colony indicated the protease positive strains which was due to hydrolysis of casein. All the three bacterial strains produced clear zone in the medium and hence was confirmed to be protease production as shown in figure 1. Culture conditions in different pH are known to influence the synthesis and secretion of extracellular enzymes by microorganisms (Sharma *et al.*, 2006; Boyoumi *et al.*, 2007; 2009; Sreenivas *et al.*, 2004). Optimization of the culture condition is necessary in the selection of the bacterial source for industrial exploitation of their extracellular enzymes. In the present investigation, skim milk powder was used as the selective substrate, in which the isolated organisms were streaked. The strains were further analyzed for protease production by fermentation process.

Enzyme activity

The objective of the present investigation was to select the bacterial strains with high level of protease producing ability. Three bacterial strains in acidic, alkaline and neutral were checked for quantitative test of extracellular protease in liquid medium and were found to produce proteases at varying levels from 55.39 IU/ml to 75.51 IU/ml at different pH 4.0, 7.0 and 10.0 (Table 1). The maximum proteolytic activity was 55.39 IU/ml at pH 4.0, 56.02 IU/ml at pH 7.0 and 75.51 IU/ml at pH 10.0 (Table 1 and Fig. 2). Several investigations have been done for screening new isolates for protease production. Previous study reported that *Bacillus anthracis* S-44 and *Bacillus cereus* S-98 exhibited their maximum ability to biosynthesize proteases within 60 h

incubation period since the productivity reached up to 126.09 units/ml for *Bacillus anthracis* S-44 corresponding to 240.45 units/ml for *Bacillus cereus* S-98 respectively (Johnvesly and Naik, 2001).

Strain identification using biochemical and morphological characterization

Table 2 indicated the morphological characteristics of the isolated strains, and Table 3 denoted the respective results of the biochemical investigations. The morphological characterization of the selected organisms revealed that they belonged to the members of *Bacillus* sp. All the three (03) strains were found to be gram positive in nature and the colonies of bacterial strains which were grown on nutrient agar plates were round with smooth, drop like/ raised/ flat, cream color and have mucilage colony and non-mucilage colony. Biofilm and pigment production around the colony were absent in all the strains. Further, the biochemical tests of Bac1, Bac2 and Bac3 confirmed the species to be *Bacillus atrophaeus*, *Bacillus cereus* and *Bacillus coagulans* respectively.

Strain identification by 16S rRNA gene sequencing

Genomic DNA of the studied strains were extracted and the yield of genomic DNA varied from 60 to 140 ng/μl. PCR amplification produced a fragment of approximately 800-1000 bp (Fig. 3). This size corresponded to the expected size as compared to other bacteria as reported by Cheng *et al.* (2010). Partial 16S rRNA gene sequences of the three (03) bacterial strains were submitted to NCBI GenBank database. A BLAST search performed on the 16S rRNA primary sequences of the three (03) bacterial strains resulted in the highest similarity score. The maximum identity

score after BLAST with 16S rRNA gene sequence of Bac1 showed 99% identity with *Bacillus* sp. KF725719, Bac2 showed 99% identity with *Bacillus* sp. KC634279 and Bac3 showed 99% identity with *Bacillus* sp. KJ801586. Accession numbers of partial 16S rRNA gene sequences of the three (03) bacterial strains were obtained as KJ562181, KM010238 and KM010243 (Table 4).

Effect of pH on protease production

The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Investigation on the proteolytic activity for high enzyme production in the strains grown in acidic, neutral and alkaline medium revealed that highest enzyme activity was found in bacterial isolates grown in alkaline medium. pH of the culture medium is important for cell growth (Li *et al.*, 2001; Singh and Das, 2011), perhaps relating to its influences on nutrient solubility and uptake, enzymatic activity, cell membrane morphology, byproduct formation and redox reactions (Bajaj *et al.*, 2009). Our finding is supported by earlier study of Anustrup (1980), and reported that the pH of the medium must be maintained above 7.5 throughout the fermentation period of the alkaline production. Zeikus *et al.* (1998) also reported that the majority of the thermophilic *Bacilli* grew at the pH range of 5.8-8.0. Johnvesly *et al.* (2002) reported the maximum enzyme activity between pH 6.5 and 12.0 indicating its potential for practical use in industrial purpose which require activity over a wide pH range. Maximum protease production was achieved by the strain *B. coagulans* KM010243 which was grown in alkaline medium followed by the strains *B. cereus* KM010238 and *B. atrophaeus* KJ562181 which were grown in neutral and acidic medium respectively (Fig. 2). Several investigations done in alkaline

protease enzyme production by *Bacillus* sp. has been previously reported by Aoyama *et al.* (2000), Feng *et al.* (2001), Adinarayana *et al.* (2003), Al-Shehri *et al.* (2004) and Schallmeyer *et al.* (2004).

Phylogenetic analysis

A total of 03 partial 16S rRNA gene sequences of investigated strains and 14 sequences from the same strains were retrieved from the NCBI GenBank database. A BLAST search performed on the 16S rRNA primary sequence resulted in the highest similarity score of the present strain studied.

The phylogenetic tree based on the NJ analysis is given in figure 4. *B. weihenstephanensis* 265ZG11 formed a cluster with strains *B. polyfermenticus* bA87

and *B. methylotrophicus* YJ11-1-4 with bootstrap values of 56%. *B. toyonensis* MCCC1A06928 was related with *B. amyloliquefaciens* GR53 with a bootstrap value of 52%. The strain *B. coagulans* KM010243 was closely related to *Bacillus* sp. F212 sharing 99% of sequence identity which topology was supported with a bootstrap value of 98%. *B. amylolyticus* SKM25 clustered with a strain of *Bacillus* sp. D5 of sequence identity supported by 75% of bootstrap values. *B. cereus* KM010238 clustered with a strain *B. subtilis* AN5 sharing 99% of sequence identity supported by 75% of bootstrap values. *B. athrophaeus* KJ562181 compared in BLAST was related to four strains, *B. megaterium* ZJ-1, *Bacillus* sp. G332, *B. thuringiensis* K206 and *Bacillus* sp. F224 supported by 89% bootstrap value.

Table.1 Quantitative enzymatic assay for protease producing *Bacillus* strains at different pH

Bacterial strain	pH	Zone of inhibition in mm	Protease (IU/ml)
Bac1 <i>B. atrophaeus</i>	4	25 mm	55.39
Bac2 <i>B. cereus</i>	7	39 mm	56.02
Bac3 <i>B. coagulans</i>	10	45 mm	75.51

Bac= Bacteria

Table.2 Morphological characteristics of bacterial strains isolated from NE region of India

Bacterial strains with NCBI accession no.	pH	Gram's nature	Colony Size	Colony configuration	Colony margin	Colony elevation	Colony color	Colony mucilage	Biofilm	Pigment production around colony
Bac1 <i>B. atrophaeus</i> (KJ562181)	4	+	5mm	Round	Smooth (entire)	Drop-like	Cream	Yes	No	No
Bac2 <i>B. cereus</i> (KM010238)	7	+	10mm	Round	Smooth	Raised	Cream	No	No	No
Bac3 <i>B. coagulans</i> (KM010243)	10	+	2mm	Round	Smooth (entire)	Flat	Cream	No	No	No

Table.3 Biochemical characteristic of the potent and investigated strains isolated from NE region of India

SN	Biochemical Test	Bac1 <i>B. atrophaeus</i>	Bac2 <i>B. cereus</i>	Bac3 <i>B. coagulans</i>
1	Malonate	-ve	-ve	-ve
2	Voges Proskauer's	+ve	+ve	-ve
3	Citrate	+ve	+ve	-ve
4	ONPG	-ve	-ve	-ve
5	Nitrate Reduction	+ve	+ve	-ve
6	Catalase	+ve	+ve	+ve
7	Arginine	-ve	-ve	+ve
8	Sucrose	+ve	+ve	+ve
9	Mannitol	+ve	-ve	-ve
10	Glucose	+ve	+ve	+ve
11	Arabinose	+ve	-ve	+ve
12	Trehalose	+ve	+ve	+ve

Table.4 Identification and sequence details of bacterial species on the basis of 16S rRNA gene sequence similarity

Bacterial strain	GenBank accession no.	Closest similarity/BLAST hit
Bac1 <i>B. atrophaeus</i>	KJ562181	KF725719
Bac2 <i>B. cereus</i>	KM010238	KC634279
Bac3 <i>B. coagulans</i>	KM010243	KJ801586

Fig.1 Protease activity on Skim Milk Agar Medium of different pH 4, 7 and 10 by *Bacillus*

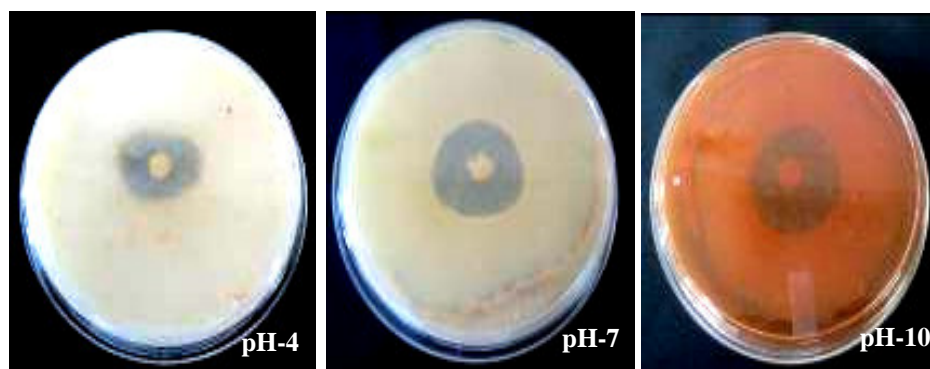


Fig.2 *Bacillus* strains showing protease activity in acidic, neutral and alkaline medium. All data are mean values of three independent measurements. Vertical bars S.D. (n=3)

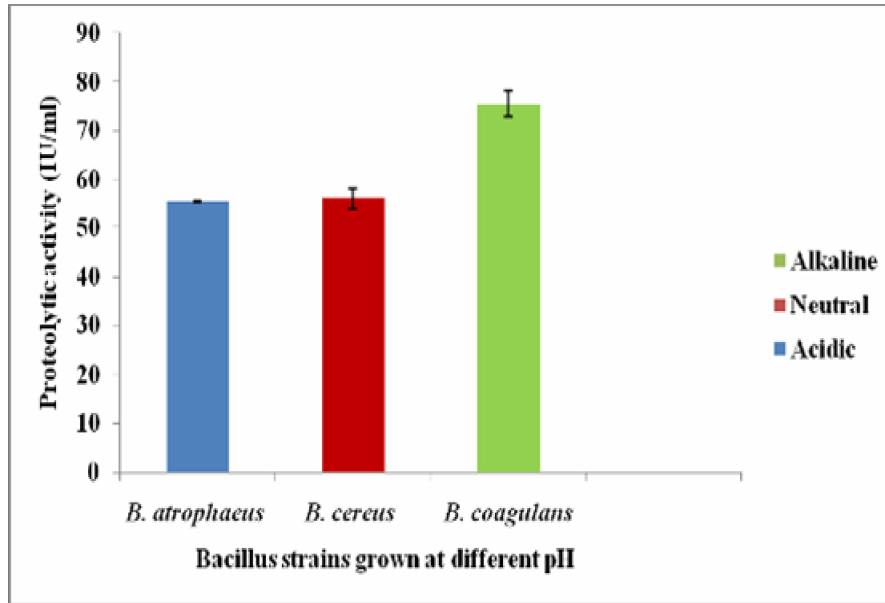


Fig.3 16S rDNA-PCR amplification of protease producing bacteria A= Bac1 B= Bac2; C=Bac3

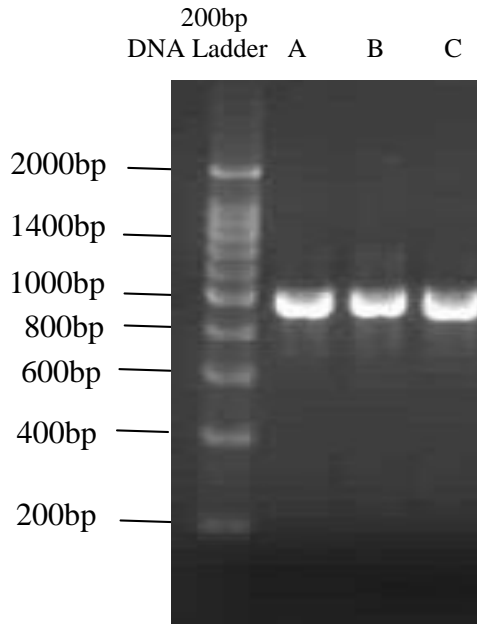
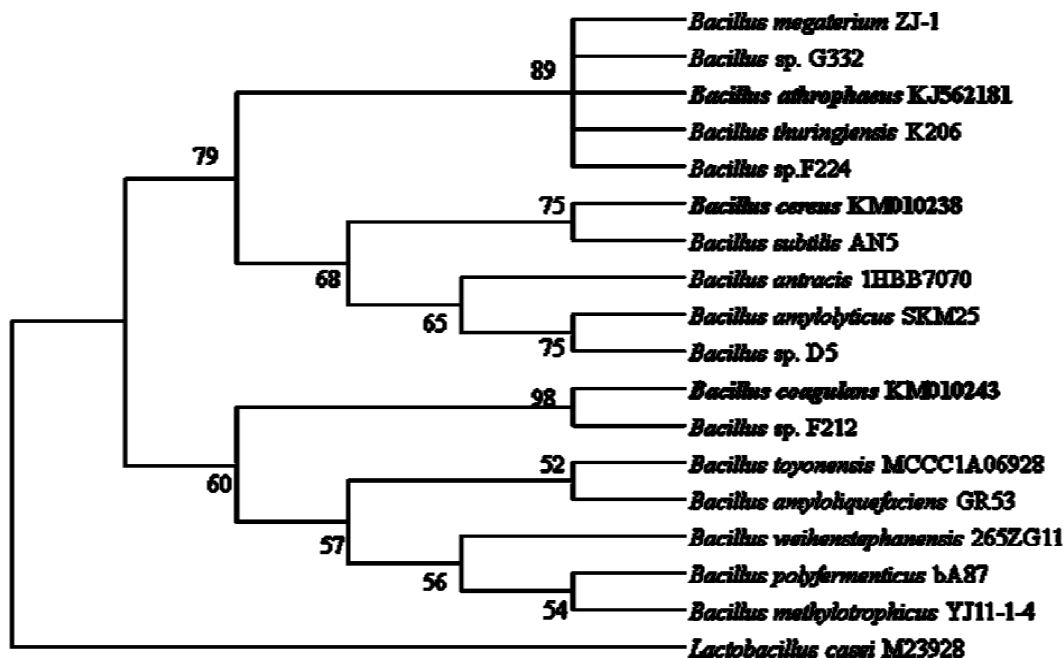


Fig.4 Phylogenetic relationships of the 03 bacterial strains investigated (in bold) and related bacteria based on 16S rDNA gene sequences. The tree was constructed using the NJ method. Percentages at nodes represent levels of bootstrap support from 1000 resampled datasets. Bootstrap values less than 50% are not shown. *Lactobacillus casei* M23928 is the outgroup taxon.



The maximum identity score after BLAST with 16S rRNA gene sequence of the present study showed 99% identity with all the *Bacillus* sp. (sequence taken from NCBI) (Table 4). Thus, lower the E-value, greater the similarity between the input sequence and the match. The clusters were well supported by bootstrap analysis and partly reflected the morphological similarity of the organisms.

Conclusion

Various bacterial isolates from soil were studied for protease producing activity. Proteolytic activity were measured for high enzyme producing strain in which the highest protease enzyme production was represented in Bac1, Bac2 and Bac3 which were grown in acidic, neutral and alkaline medium. However, the production was

highly observed in Bac3 grown in alkaline medium. Morphological and biochemical analysis revealed the bacterial strains as group of *Bacillus* sp. Moreover, molecular identification by 16S rRNA gene sequences proved that the studied strains as novel *Bacillus* strains. Hence, the results presented here suggested that high protease production can be expected with the selection of appropriate bacterial strains cultured under optimum conditions, which can be used for various application purposes in detergent industries, food industries and pharmaceutical industries.

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Conflict of interest

The authors declare that there is no conflict of interest.

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