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Screening of Cultivable Protease-Producing Bacteria in Marshy Sediments of Mangrove Forests, Goa, India

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ABSTRACT

Mangroves, the coastal wetland forests are the natural inhabitants of variety of microbes especially halophilic bacteria. These halophilic bacteria are prevalent in ocean sediments and play crucial roles in degradation of sedimentary nitrogenous organic materials. The halophilic microorganisms are loaded with potential hydrolytic enzymes to be used in industrial and/or in many biotechnological processes. The bacterial diversity of mangroves in India and variety of their proteases inhabitants are largely unknown. This ambiguity is ascribed to enclosed ecosystem of mangrove region. This study is an attempt to unearth the expediency of mangrove forest for their variety of proteases. For this, bacterial samples were collected from Marshy Sediments of Mangrove Forests, Goa, India and were screened for proteases. Twenty isolates exhibited the proteolytic activity on Casein agar and Gelatin agar plates containing 3% (w/v) NaCl. All the isolates were identified by morphological and molecular characteristics. 16S rRNA sequence analysis showed their homology to Bacillus sp. Qualitative and quantitative enzyme essays revealed potential proteases from B2 and B3 bacterial strains. This study provided novel insights on the community structure of cultivable protease-producing bacteria in the marshy areas of Mangrove forests of Goa.

Keywords: Proteases, Protease-Producing Bacteria, Marshy Land, Mangroves, 16S Rrna Sequencing.

I. INTRODUCTION

Proteases are a large group of proteolytic enzymes that are ubiquitous in nature. These are omnipresent, found in a wide diversity of sources such as plants, animals and microorganisms. Microbial proteases are the most important industrial enzymes accounting for around 60% of the total industrial enzyme market and account approximately 40% of the total enzyme production in the world (Ng and Kenealy, 1986; Rao *et al.*, 1998; Horikoshi, 1999; Gupta *et al.*, 2002). On the basis of their optimal hydrolytic activity pH range, Proteases are classified as alkaline, acid and neutral. Among those proteases, the alkaline ones are particularly relevant due to their activity and stability in high pH value (Rao *et al.*, 1998; Gupta *et al.*, 1999, 2002a, 2002b) and, consequently, their wide biotechnological potential for industrial sectors such as laundry detergents, leather tanning, beer, food and pharmaceutical industry (Rao *et al.*, 1998; Schallmey *et al.*, 2004; Fujinami and Fujisawa, 2010). Currently, a large number of commercially available alkaline proteases are extracted from *Bacillus* strains (Rao *et al.*, 1998; Gupta *et al.*, 1999, 2002a, 2002b; Schallmey *et al.*, 2004; Fujinami and Fujisawa, 2010). Over the years, *Bacillus* species as well as *Bacillus*-related genera have emerged as promising extracellular protease producers (Schallmey *et al.*, 2004; Fujinami and Fujisawa, 2010). Extracellular proteases from moderately halophilic microorganisms have been thoroughly identified and characterized in many studies (Joo *et al.*, 2003; Setyorini *et al.*, 2006; Alvarez *et al.*, 2006; Namwong *et al.*, 2006; Akolkar *et al.*, 2008; Phrommao *et al.*, 2011 and Karan and Khare, 2011).

Mangroves are the coastal wetland forests found around the world near intertidal regions of estuaries between creeks, lagoons and marshes and are rich in carbon and other nutrients. They provide a unique ecological site to diverse microbial communities' *viz.* bacteria, fungi and actinomycetes. Halophilic bacterial community includes nitrogen fixing bacteria, phosphate solublising bacteria, Sulphate reducing bacteria, photosynthetic anoxygenic bacteria, methanogenic bacteria and enzyme producing

bacteria (K.Sahoo and N.K.Dhal, 2009). Mangrove ecosystem engraves diversity of proteases producing microbes and can be exploited as a potential commercial source of proteases.

Deeming the importance of proteases, it is vital to produce them commercially. Thus, it is necessitated to look for some cultivable commercial sources of proteases. The present study was conducted with the aim of screening sediment samples from mangroves forest for efficient protease-producing bacterial sp. Further our study was aimed at determining the optimal production conditions of these proteases. Thus, plating with heat-shocked macerated samples on agar, Zobell marine agar and Casein agar media resulted in two efficient proteolytic bacterial isolates which were later identified as *Bacillus* species on the basis of their morphological and biochemical characteristics.

II. MATERIAL AND METHODS

Collection of Sediment Samples

For screening of protease producing microorganisms, Mangrove sediment samples (B1-B20) were from Fresh Water Lake (FWL) and Mangrove forest (M) areas in the vicinity of Dr. Salim Ali Bird Century, Ribandar Ferry, Chorao, (15°, 30', 27" N and 73°, 51', 36" E), Goa, India in July, 2015. The sediment samples were collected using a Suspended Sediment Sampler (Kaizen, India) in sterile, zipped plastic sample bags (Tarsons, India) pre cooled at 0°C. The samples were transported back to laboratory on the same day in liquid nitrogen (-78 °C) and immediately frozen in -80 °C till further use. Surface temperature and pH of sediment site were detected in situ using a pH meter

Isolation of Microorganisms

The microorganisms were isolated by using standard spread plate technique as described by (Zhou et al., 2009). Briefly, 1 g (fresh wet weight, in triplicate) of all 20 collected sediment samples were weighed equally before mixing and were serially diluted to 10^{-6} with artificial sea water (collected from the same site). 100µl of all the triplicate aliquots of the serially diluted samples (10^{-1}–10^{-6}) were spread separately on the screening plates composed of 0.2% (w/v) yeast extract, 0.3% (w/v) casein, 0.5% (w/v) gelatin, 1.5% (w/v) agar powder, in 1 L artificial seawater at pH 8.0. All the plates with inoculum were incubated at 25^0 C until colonies with clear hydrolysis zones were detected (3 weeks). Colonies were selected on the basis of different morphological characteristics (e.g., colony color, size and surface polysaccharides) and further purified by repeated streaking on the same plate until uniform or pure colonies were observed (Table.1.). Pure cultures obtained from repetitive cultures were preserved at −80˚C in 20% (v/v) glycerol till further use.

Qualitative Estimation or Detection of Protease Activity

The isolated bacterial cultures were screened and checked qualitatively for their ability for protease production as described by Zhou *et al*., 2009. In brief, the 20 isolated bacterial cultures were inoculated on Casein agar and Gelatin agar plates. The media was then incubated at room temperature for 24 hours. The plates were stained with TCA (5 %) for 5 minutes and observed for clear zone on the turbid protein precipitated background.

Quantitative Protease Activity

Quantitative protease activity in the crude enzyme extract was determined according to the method as described by Carrie CuppEnyard (2008) by using casein as a substrate. Briefly, 5 mL of 0.65% casein solution was taken in two test tubes (sample-S, Test-T) and incubated for 5 at 37 ˚C for 10 min. One mL of enzyme solution was added in T-test tube. It was mixed properly and incubated at 37 ˚C in a water bath for 30 min for allowing the enzymatic reaction to occur. The reaction was terminated by adding 5 mL of 1% Trichloroacetic acid (TCA) solution to both test and blank tubes. One mL of enzyme solution was further added in blank test tube and was incubated for 15 minutes at room temperature. Solution from both test tubes was filtered using Whatmann's No 1 filter paper. Two mL of test and blank filtrate were taken in two new test tubes and labeled as test (T) and blank (B). Five mL of sodium carbonate was added in both test tubes followed by addition of 1 mL of 2 fold diluted Folin Ciocalteus phenol reagent. The resulting solutions in both test tubes were incubated in dark for 30 minutes at room temperature for the development of blue color. The

absorbance of the blue color compound was measured at 660 nm against a reagent blank using tyrosine standard. One protease unit was defined as the amount of enzyme that releases 1 µM of tyrosine per minute at pH 7.5 at 37 ºC (Mohapatra et al., 2003).

16S rRNA Gene Amplification and Phylogeny

For 16S rRNA gene amplification, all 20 bacterial isolates were first incubated in the liquid medium (screening media without agar) at 25^0 C with shaking of 300 rpm for 15 min. The biomass of each bacterial isolate was collected through centrifugation. Genomic DNA Extraction was carried out using Uniflex DNA Isolation Kit (GeNei, 612117000051730), following the manufacturer's instructions. Both DNA quality and quantity were determined by using Quant-iT ™ dsDNA BR Assay Kit (Invitrogen, USA) in Qubit ™ fluorometer. 2 µl of triplicates Bacterial Samples were quantified and the results (µg/ml) are summarized in table 2 below.

The 16S rRNA gene of all the isolates was amplified using Biometra thermal cycler (T-Personal 48) and sequenced from genomic DNA using universal primers **(5'-3');** 519F (Forward, CAGCAGCCGCGGTAATAC, Bioserve Biotechnologies Pvt. Ltd., India) and 1385R (Reverse, CGGTGTGTACAAGGCCC, Bioserve Biotechnologies Pvt. Ltd., India) as described by Ute Hentschel et al. 2002 (Fig.1).

The amplified PCR product was purified using AxyPrep PCR Clean up kit (Axygen, AP-PCR-50) and sent for DNA sequencing by Sanger method (Sanger et al., 1977). Briefly, 100µl of PCR-A buffer was mixed to the 25µl of reaction mixture and transferred to a column placed in a 2ml collection tube and centrifuged (10,000 rpm, 1min, 0° C). The filtrate was discarded and 700µl of W2 buffer was added to the column and further centrifuged (10,000rpm, 2min, and 0°C). The column was then transferred to a new tube. 25µl of Eluent was added into the column, incubated at RT for 2min and centrifuged at 10,000rpm for 5min. It was then loaded on an Applied Biosystems 3730xl DNA analyzer (Thermo Fisher, USA) and chromatogram was obtained. The sequencing primer used was 519F: 5'CAGCAGCCGCGGTAATAC3'. Identification of the sequences generated in this study was carried out by searching for their most similar sequences in the NCBI GenBank using the BLASTn approach. The BLAST results were used to find out evolutionary relationship of isolates. Then, a 16S rRNA gene Phylogenetic tree (fig. 7&8) was reconstructed using the neighbor-joining method (Saitou and Nei, 1987) with Kimura's two-parameter model in MEGA version 5.05 (Tamura et al., 2011).

III. RESULTS AND DISCUSSION

Results

Morphology Studies of the Bacterial Colonies

All 20 bacterial isolates were studied for their morphological characteristics *viz.* shape, size color and their culture media. The results are summarized in Table.1. Morphological studies showed that size varied from 1 mm to 8 mm. Colors of the bacterial suspension varied from cream, light brown, white orange and yellow. Their consistency was either opaque and or found translucent.

Qualitative Protease Activity

All 20 bacterial cultures were checked for their qualitative protease activity using Casein agar and Gelatin agar plates. Zones of clearance of all 20 bacterial isolates were observed and summarized in Table. 2. It is elucidated from the table that 15 cultures out of 20 were positive for Protease production. Out of these 15 cultures, 05 showed only Gelatinase activity while 02 showed only Caseinase activity (Table.2). The remaining cultures gave zone of clearance on both substrates i.e. Milk agar and Gelatin agar plates. Bacterial isolates B2 and B3 showed potential protease activity as illustrated in Figures 2a & 2b. Qualitative analysis clearly depicted that B2 and B3 was found to be persuasive protease producing bacterial strains in both casein and Gelatin agar mediums.

Quantitative Protease Activity

Quantitative protease activity in the crude enzyme extract was determined according to the method as described by Carrie CuppEnyard (2008) by using casein as a substrate. All the experiments were done in triplicates and mean value was presented in Fig.3.Quantitative proteolytic analysis showed that B3 bacterial strain was most proficient in its proteolytic activity followed by B2. Data further proposed that bacterial strains B15, B18, B6 and B14 also showed normal proteolytic activities and can serve as potential proteolytic enzyme sources.

Phylogenetic Analysis of Microbes Producing Enzyme by 16srRNA Technique

For Phylogenetic association studies, DNA of B2 and B3 was amplified and an optimal concentration of B2 (50µg/ml) and that of B3 (80µg/ml) was used. 16S rRNA genes of the B2 and B3 isolates were amplified and optimally sequenced. 16S rRNA gene sequences were for finding the Phylogenetic association between the species. A distance-based neighbor-joining Phylogenetic tree was constructed using the sequences from this study using MEGA version 5.05 by taking reference sequences from the GenBank database. Phylogenetic analysis confirmed B2 as *Bacillus anthracis* and B3 as *Bacillus safensis* respectively. Phylogenetic association of B2 is shown in Table 3 and Fig.4. Phylogenetic position of B3 is depicted in Table 4 and Fig.5.

Phylogenetic Identification of B2

AGGAACGTTAACGGATTATTGGGCGTAAGCGC
GCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGC
CCACGGCTCAACCGTGGAGGGTCATTGGAAACT
GGGAGACTTGAGTGCAGAAGAGGAAAGTGGAA
TTCCATGTGTAGCGGTGAAATGCGTAGAGATAT
GGAGGAACACCAGTGGCGAAGGCGACTTTCTG
GTCTGTAACTGACACTGAGGCGCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGAGTGCTAAGTGTTAGA
GGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCA
TTAAGCACTCCGCCTGGGGAGTACGGCCGCAA
GGCTGAAACTCAAAGGAATTGACGGGGGCCCG
CACAAGCGGTGGAGCATGTGGTTTAATTCGAAG
CAACGCGAAGAACCTTACCAGGTCTTGACATCC
TCTGACAACCCTAGAGATAGGGCTTCTCCTTCG
GGAGCAGAGTGACAGGTGGTGCATGGTTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCC
CGCAACGAGCGCAACCCTTGATCTTAGTTGCCA
TCATTAAGTTGGGCACTCTAAGGTGACTGCCGG
TGACAAACCGGAGGAAGGTGGGGATGACGTCA
AATCATCATGCCCCTTATGACCTGGGCTACACA
CGTGCTACAATGGACGGTACAAAGAGCTGCAA
GACCGCGAGGTGGAGCTAATCTCATAAAACCG
TTCTCAGTTCGGATTGTAGGGCTGCAACTCGCC
TACATGAAGCTGGAATCGCTAGTAATCGCGGAT
CAGCATGCCCGCGGTGAATACGTTCCCGGGCTG
TACACCCCACCGAGAGAGA (Sequence length: 865bp)

Phylogenetic Identification of B3

CGGGATCGGGTGGCTCGGAATTATTGGGCGTAA
GGGCTCGCAGGCGGTTTCTTAAGTCTGAT
TGAAAGCCCCCGGCTCAACCGGGGAGGGTCAT
TGGAAACTGGGAAACTTGAGTGCAGAAGA
GGAGAGTGGAATTCCACGTGTAGCGGTGAAAT
GCGTAGAGATGTGGAGGAACACCAGTGGCGAA
GGCGACTCTCTGGTCTGTAACTGACGCTGAGGA
GCGAAAGCGTGGGGAGCGAACAGGATTAGATA
CCCTGGTAGTCCACGCCGTAAACGATGAGTGCT
AAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGC
AGCTAACGCATTAAGCACTCCGCCTGGGGAGTA
CGGTCGCAAGACTGAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTT
TAATTCGAAGCAACGCGAAGAACCTTACCAGG
TCTTGACATCCTCTGACAACCCTAGAGATAGGG
CTTTCCCTTCGGGGACAGAGTGACAGGTGGTGC
ATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGATC
TTAGTTGCCAGCATTCAGTTGGGCACTCTAAGG
TGACTGCCGGTGACAAACCGGAGGAAGGTGGG
GATGACGTCAAATCATCATGCCCCTTATGACCT
GGGCTACACACGTGCTACAATGGACAGAACAA
AGGGCTGCAAGACCGCACGTTTAGCCAATCCCA
TAAATCTGTTCTCAGTTCGGATCGCAGTCTGCA
ACTCGACTGCGTGAAGCTGGAATC
GCTAGTAATCGCGGATCAGCATGCCGCGGTGG
AATACGTTCCCGGGCTGTTGACCCCCCGCGAGA
GGA (Sequence length: 868bp).

Discussion

The mangrove region in Goa is the largest mangrove forest area in India and known for its high biodiversity and abundance of plants, animals and microorganisms. It is frequently studied for its rich microbial diversity which is an inhabitant of variety of proteolytic enzymes (Bowman. J.P. et al., 2003; Chen .X.L. et al., 2003; Dajanta K. et al., 2009 and Gerday C. et al., 2000). Worldwide, many researchers found alkalophilic bacterial species *viz. Bacillus subtilis, Bacillus pantothenticus, Bacillus circulans, Bacillus brevis, Serratia liquefaciens, Chromobacterium violaceum and Pseudomonas aeruginosa* as rich sources of proteolytic enzymes and further attributed these enzymes as pivotal industrial enzymes (Gray J.P. and Herwig R.P., 1996; Gupta, N., et al., 2007; Hanan S.A. 2012; Hunter E.M., et al., 2006; Lerson H. 2001). The present study also communicates similar line of thought. All twenty types of halotolerant bacteria isolated from Mangrove areas were screened for their proteolytic enzymes and their

efficacy. Similar results were also achieved by Dajanta et al., 2009 and Taprig et al., 2013. Among all the isolates, five isolated bacteria exhibited potential proteolytic activity with the clear zone diameter of more than 10 mm. Out of the five; two bacterial isolates; B3 and B2 were found to be most promising in their proteolytic activities. The Phylogenetic analysis of B2 and B3 confirmed these isolates as *Bacillus anthracis* and *Bacillus safensis* respectively. Phylogenetic association of B2 is shown in Table 3 and Fig.4. Phylogenetic position of B3 is depicted in Table 4 and Fig.5. These B2 and B3 strains were used for isolation of proteolytic enzymes which were found to be potent proteases. These Proteases currently used in industry fields belong to *Bacillus* species, such as *B. Subtilis, B. cereus, B. licheniformis, B. megaterium, B. halodurans* and *B. alkaliphilus* (Maurer, 2004, Hanan, 2012). Thus, the present research is obliging in terms of some novel bacterial strains for finding new series of proteases which reveals their usefulness in industrial applications.

IV. CONCLUSION

Mangroves are unique swampy regions with water being alkaline in nature and sediment or soil region having a neutral to slightly acidic pH. Since mangrove environment is prevalent to salt stress conditions, microorganisms growing under such stress conditions could have a potential for bioremediation programmes. The soil isolates were halo-tolerant and could tolerate relatively high concentrations of polluted stuffs. Mangroves are saline coastal ecosystem rich in Carbon and other nutrients. They harbor large numbers of population of unique bacteria. The present study reveals the mixed population of bacteria of Goa region in India. Further studies on commercial exploitation of these organisms and more evaluation of their stress tolerance could make them applicable for various industrial applications.

V. Conflicts of Interests

Author shows no conflicts of interests.

VI. Acknowledgement

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Table 1 : Morphological characteristics of bacteria isolated from Marshy Sediments of Mangrove Forests, Goa, India

Culture Code	Shape	Size	Consistency	Color	Media*	Source**
B1	Rough Edge Raised	1mm	Opaque	White	ZMA	MS
B2	Round Smooth Raised	3mm	Opaque	Off-white	CA	FWL
B3	Watery Smooth Uneven edge	8mm	Translucent	Milky	NA	FWL
B4	Flat Rough edge	2mm	Translucent	Brown	ZMA	MS
B5	Smooth edge Flat	3mm	Opaque	Off-white	ZMA	MS
B6	Raised Smooth edge	4mm	Opaque	Cream	CA	FWL
B7	Smooth edge Flat	2mm	Opaque	Yellow	NA	FWL
B8	Raised Smooth edge	4mm	Opaque	Light Brown	ZMA	FWL
B9	Smooth edge Flat	2mm	Translucent	Colorless	NA	FWL
B10	Rough edge Raised	3mm	Opaque	Brown	CA	FWL
B11	Flat Rough edge	5mm	Transparent	White	NA	FWL
B12	Smooth edge Flat	4mm	Opaque	Orange	ZMA	MS
B13	Rough edge Raised	4mm	Opaque	White	ZMA	MS
B14	Flat Rough edge	3mm	Translucent	Off-white	CA	MS
B15	Rough edge Raised	2mm	Opaque	White	CA	FWL
B16	Sticky Smooth edge Flat	3mm	Opaque	Yellow	ZMA	MS
B17	Slimy Uneven edge	5mm	Translucent	Colorless	CA	FWL
B18	Raised, Smooth edge	4mm	Opaque	Off-white	CA	FWL
B19	Smooth edge Flat	1mm	Translucent	Red	ZMA	MS
B20	Rough edge Raised	3mm	Opaque	Colorless	NA	MS

Media*: Zobell Marine Agar (ZMA); Nutrient Agar (NA); Casein Agar (CA).
Source**: Mangrove: Swampy (M) Fresh Water Lake (FWL).

Table 2: Diameter of Zone of clearance for isolated bacteria

Culture Code	Zone of Clearance (mm)	
	Casein Agar	Gelatin Agar
B1	--	**5**
B2	**20**	**15**
B3	**20**	**25**
B4	--	--
B5	--	**3**
B6	**10**	**8**
B7	--	**6**
B8	--	--
B9	**7**	--
B10	**15**	**5**
B11	**12**	**15**
B12	--	--
B13	--	--
B14	**5**	--
B15	**15**	**15**
B16	--	--
B17	--	**5**
B18	**10**	**12**
B19	--	**6**
B20	**..**	**6**

Table 3: Phylogenetic neighbors of B2 based on 16S rRNA gene sequence

Description	Max score	Query cover	E value	Ident	Accession
Bacillus toyonensis strain BCT-7112 16S ribosomal RNA gene, complete sequence	1515	97%	0.0	99%	NR_121761.1
Bacillus thuringiensis Bt407 16S ribosomal RNA, complete sequence	1515	97%	0.0	99%	NR_102506.1
Bacillus anthracis str. Ames strain Ames 16S ribosomal RNA, complete sequence	1515	97%	0.0	99%	NR_074453.1
Bacillus cereus ATCC 14579 16S ribosomal RNA (rRNA) gene, complete sequence	1515	97%	0.0	99%	NR_074540.1
Bacillus cereus strain JCM 2152 16S ribosomal RNA gene, partial sequence	1515	97%	0.0	99%	NR_113266.1
Bacillus thuringiensis strain NBRC 101235 16S ribosomal RNA gene, partial sequence	1515	97%	0.0	99%	NR_112780.1
Bacillus cereus strain CCM 2010 16S ribosomal RNA gene, complete sequence	1515	97%	0.0	99%	NR_115714.1
Bacillus cereus strain NBRC 15305 16S ribosomal RNA gene, partial sequence	1515	97%	0.0	99%	NR_112630.1
Bacillus cereus strain ATCC 14579 16S ribosomal RNA gene, partial sequence	1515	97%	0.0	99%	NR_114582.1
Bacillus thuringiensis strain ATCC 10792 16S ribosomal RNA gene, partial sequence	1515	97%	0.0	99%	NR_114581.1
Bacillus thuringiensis strain IAM 12077 16S ribosomal RNA gene, partial sequence	1515	97%	0.0	99%	NR_043403.1

Bacillus cereus strain IAM 12605 16S ribosomal RNA gene, partial sequence	1515	97%	0.0	99%	NR_115526.1
Bacillus pseudomycoides strain NBRC 101232 16S ribosomal RNA gene, partial sequence	1509	97%	0.0	99%	NR_113991.1
Bacillus weihenstephanensis KBAB4 strain KBAB4 16S ribosomal RNA, complete sequence	1504	97%	0.0	99%	NR_074926.1
Bacillus mycoides strain NBRC 101228 16S ribosomal RNA gene, partial sequence	1504	97%	0.0	99%	NR_113990.1
Bacillus mycoides strain ATCC 6462 16S ribosomal RNA gene, partial sequence	1504	97%	0.0	99%	NR_115993.1
Bacillus weihenstephanensis strain DSM 11821 16S ribosomal RNA gene, partial sequence	1504	97%	0.0	99%	NR_024697.1
Bacillus mycoides strain 273 16S ribosomal RNA gene, partial sequence	1504	97%	0.0	99%	NR_036880.1
Bacillus marcorestinctum strain LQQ 16S ribosomal RNA gene, partial sequence	1489	96%	0.0	99%	NR_117414.1
Bacillus anthracis strain SB1 16S ribosomal RNA gene, partial sequence	1487	95%	0.0	99%	NR_118379.1

Table 4 : Phylogenetic neighbors of B3 based on 16S rRNA gene sequence

Description	Max score	Query cover	E value	Ident	Accession
Bacillus safensis strain NBRC 100820 16S ribosomal RNA gene, partial sequence	1515	95%	0.0	99%	NR_113945.1
Bacillus safensis strain FO-36b 16S ribosomal RNA gene, partial sequence	1515	95%	0.0	99%	NR_041794.1
Bacillus pumilus SAFR-032 strain SAFR-032 16S ribosomal RNA, complete sequence	1504	95%	0.0	99%	NR_074977.1
Bacillus aerius strain 24K 16S ribosomal RNA gene, partial sequence	1504	95%	0.0	99%	NR_118439.1
Bacillus stratosphericus strain 41KF2a 16S ribosomal RNA gene, partial sequence	1504	95%	0.0	99%	NR_042336.1
Bacillus pumilus strain NBRC 12092 16S ribosomal RNA gene, partial sequence	1504	95%	0.0	99%	NR_112637.1
Bacillus altitudinis strain 41KF2b 16S ribosomal RNA gene, partial sequence	1504	95%	0.0	99%	NR_042337.1
Bacillus pumilus strain ATCC 7061 16S ribosomal RNA gene, partial sequence	1504	95%	0.0	99%	NR_043242.1
Bacillus stratosphericus strain 41KF2a 16S ribosomal RNA gene, partial sequence	1498	95%	0.0	99%	NR_118441.1
Bacillus pumilus strain SBMP2 16S ribosomal RNA gene, partial sequence	1483	95%	0.0	99%	NR_118381.1
Bacillus atrophaeus strain NBRC 15539 16S ribosomal RNA gene, partial sequence	1472	95%	0.0	99%	NR_112723.1
Bacillus atrophaeus 1942 strain 1942 16S ribosomal RNA, complete sequence	1471	95%	0.0	99%	NR_075016.1
Bacillus atrophaeus strain JCM 9070 16S ribosomal RNA gene, partial sequence	1471	95%	0.0	99%	NR_024689.1
Bacillus subtilis strain 168 16S ribosomal RNA gene, complete sequence	1454	95%	0.0	98%	NR_102783.1
Bacillus amyloliquefaciens subsp. *plantarum*	1454	95%	0.0	98%	NR_075005.1

strain FZB42 16S ribosomal RNA gene, complete sequence					
Bacillus subtilis subsp. *spizizenii* strain ATCC 6633 16S ribosomal RNA gene, partial sequence	1454	95%	0.0	98%	NR_118486.1
Bacillus subtilis strain SBMP4 16S ribosomal RNA gene, partial sequence	1454	95%	0.0	98%	NR_118383.1
Bacillus vallismortis strain NBRC 101236 16S ribosomal RNA gene, partial sequence	1454	95%	0.0	98%	NR_113994.1
Bacillus subtilis subsp. *inaquosorum* strain BGSC 3A28 16S ribosomal RNA gene, partial sequence	1454	95%	0.0	98%	NR_104873.1
Bacillus tequilensis strain 10b 16S ribosomal RNA gene, partial sequence	1454	95%	0.0	98%	NR_104919.1

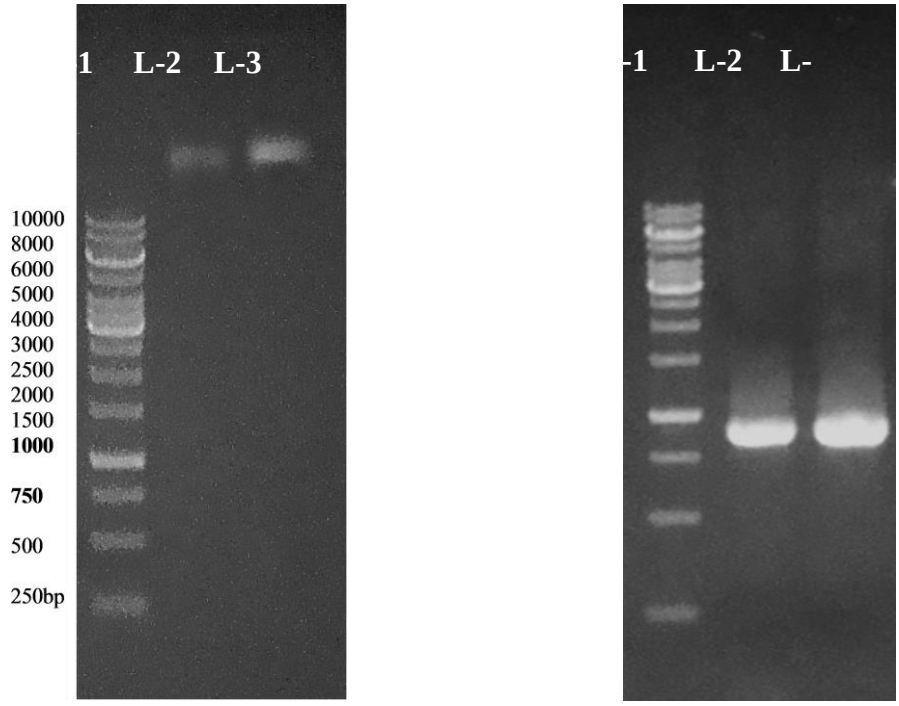
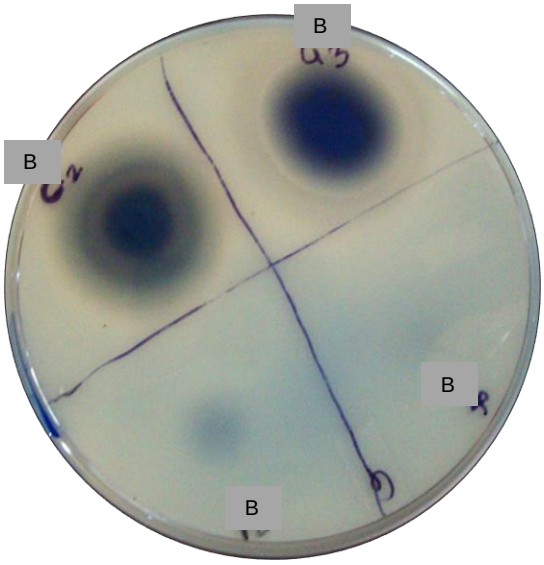


Fig. 1a: Extraction of Genomic DNA from B2 and B3 strains of bacterial samples procured from mangrove forest regions in Goa, India. Lane 1: DNA marker from fermentas (SM#0311); Lane2: Genomic DNA from B2; Lane3: Genomic DNA from B3;

Fig. 1b: Amplified Genomic DNA from B2 and B3 strains of bacterial samples procured from mangrove forest regions in Goa, India. Lane 1: DNA marker from fermentas (SM#0311); Lane2: amplified genomic DNA from B2; Lane3: amplified genomic DNA



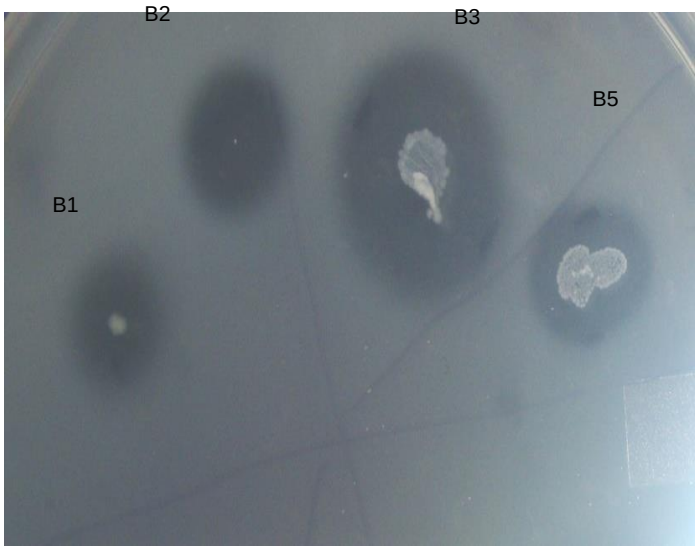


Fig. 2a. Figure depicts qualitative estimation of protease activity of two potential strains. Qualitative activity is measured in terms of Zone of clearance around colonies on Casein Agar plate; **Fig. 2b**. Fig shows Zone of clearance around colonies on Gelatin Agar plate.

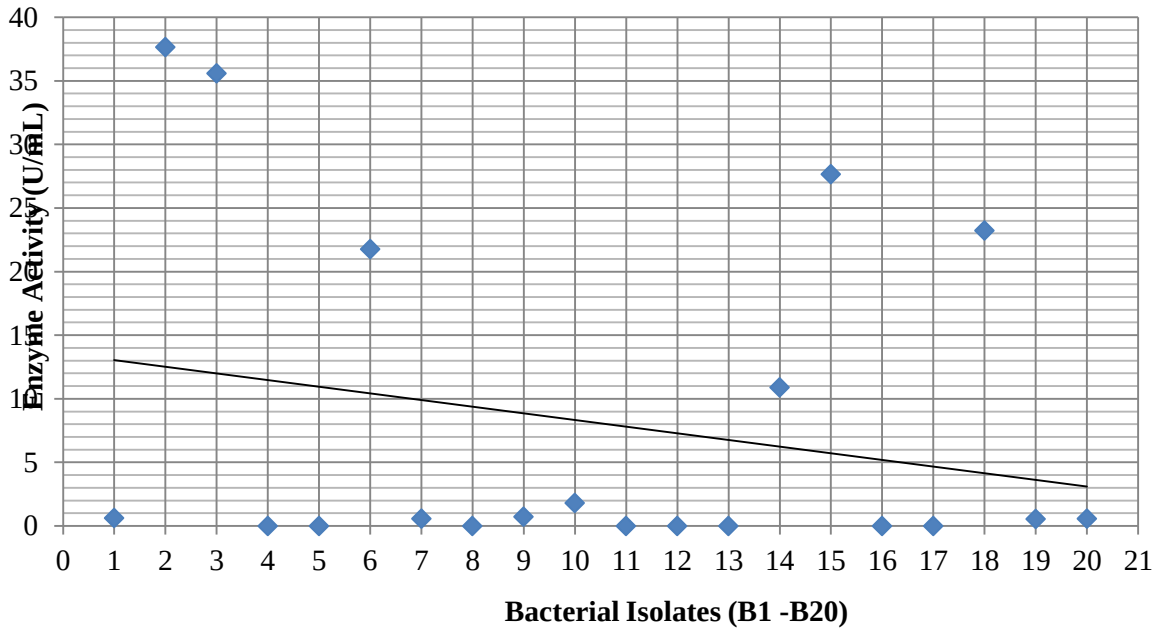


Fig. 3. Figure illustrates the quantitative protease analysis as shown by all 20 bacterial isolates. Quantitative activity is expressed in mean OD at 660 nm using tyrosine as a standard. One protease unit was defined as the amount of enzyme that releases 1 µM of tyrosine per minute at pH 7.5 at 37 ºC

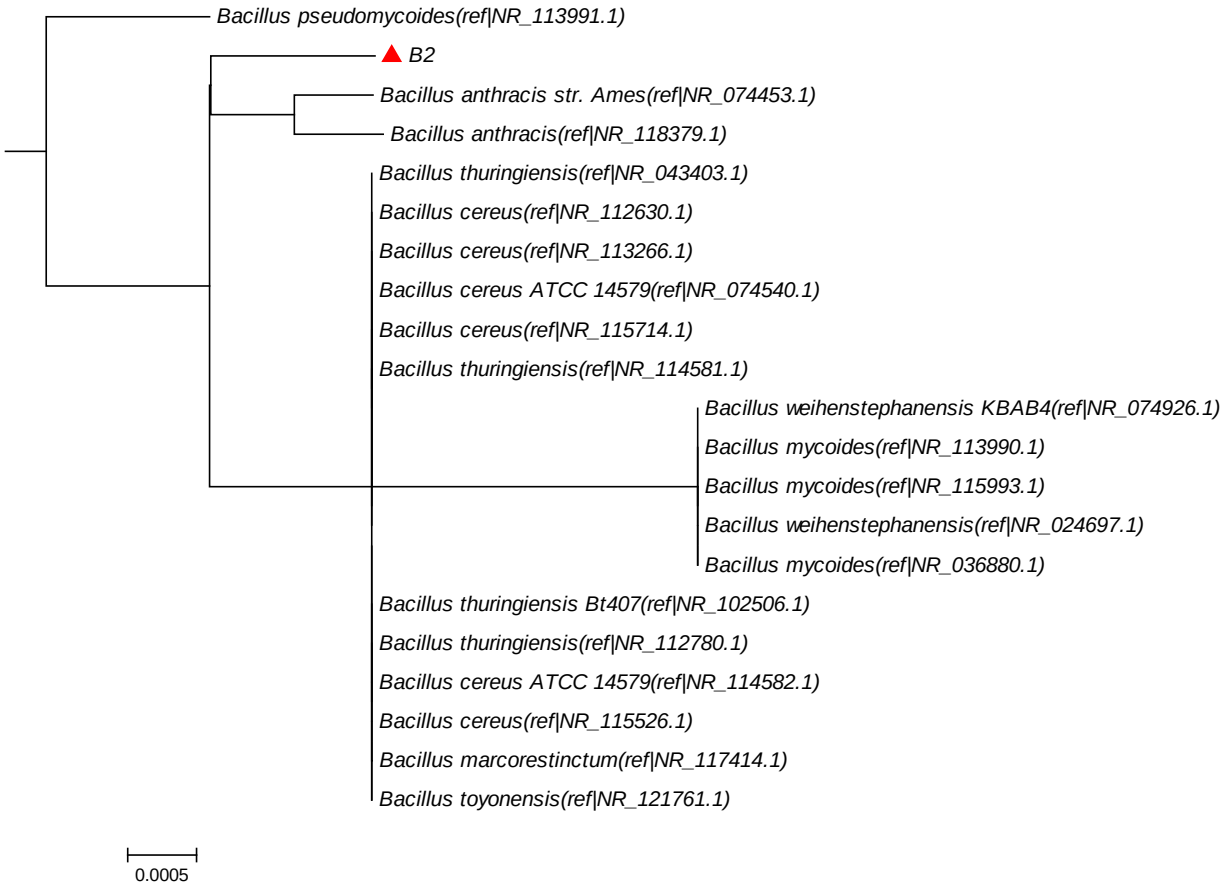


Fig. 4: Phylogenetic tree for B2 using 16S rRNA gene sequence

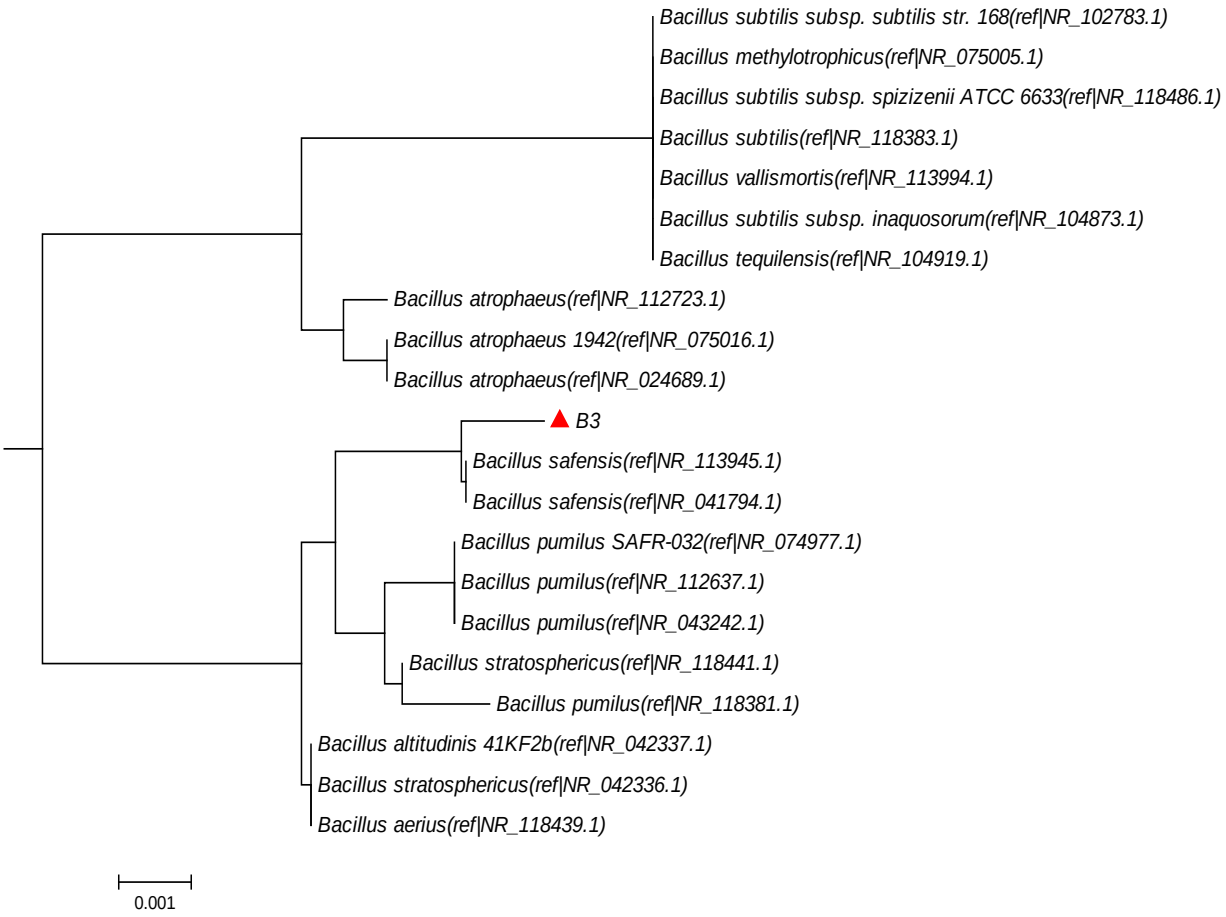


Fig. 5: Phylogenetic tree for B3 using 16S rRNA gene sequence