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ISOLATION, SCREENING AND IDENTIFICATION OF PECTINASE-PRODUCING BACTERIAL STRAIN *BACILLUS CEREUS* FROM MARINE SEDIMENT SAMPLES

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ABSTRACT: Marine environment is an enormous pool of biodiversity resources that cover approximately 70% surface of the earth. Marine microorganisms have unique properties since they have to adapt to extreme marine environmental conditions in deep-sea water. The present study aims to isolate, screen and identify bacterial strains from marine sediment samples that produce an enzyme pectinase with clinical and industrial applications. Marine sediment samples were collected and cultured on zobell marine agar medium. After incubation, five bacterial strains were screened for pectinase activity. Two isolates showed pectinase activity on Yeast Extract Pectin (YEP) medium. Morphological and biochemical characteristics of pectinase-producing organisms were done. Pectinase-producing bacterial strain (PE S7C) was selected based on their pectinase test. The selected strain PES7C was further sequenced for identification. The phylogenetic characterization and 16S rRNA of the strain PE S7C revealed that bacterial cultures belong to *Bacillus cereus*. The morphological studies indicated that the isolate was Gram $_{+}$ve, rod-shaped and motile organism. The present study concludes that marine bacteria can be a source of pectinase with important applications.

INTRODUCTION: The marine environment is the largest habitat on earth. Oceans include the greatest extremes of temperature, light, and pressure encountered by life [1]. Adapting marine bacteria to harsh environments has led to a rich biological and genetic diversity. Marine bacteria are attracting attention as new biotechnological resources. These marine bacteria can be a potential source of new bioactive compounds for industrial, agricultural, environmental, pharmaceutical, and medical uses [2]. Marine microbes are tiny single-celled organisms that live in the ocean and account for more than 98 percent of ocean biomass.

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Marine microorganisms are a valuable source of novel enzymes with ideal characteristics because of the halophilic nature of marine bacteria [3]. Moreover, marine microorganisms are reported to produce enzymes with industrially important properties, such as stability at elevated temperatures and alkaline pH conditions [4]. Marine organisms represent around 50% of the worldwide biodiversity [5]. In addition to their chemical and genetic diversities, they represent a potential source of broad spectrums of commercially valuable and diverse product, such as polysaccharides, enzymes, peptides, lipids, steroids and terpenoids [6].

Marine enzymes have great biotechnological and industrial applications in pharmaceuticals, foods, textile, beverage product, agricultural, chemical, and biomedical sectors [7, 8]. Marine microbial enzymes have wide applications in bioindustries [9, 10]. Microbial enzymes have a great number of uses in food, pharmaceutical, textile, paper, leather, and

other industries [11]. Pectinases are a group of enzymes that degrade pectic substances and are classified according to their mechanism of action [12, 13]. Studies have reported that pectinase of microbial origin accounts for 25% of global food and industrial enzyme sales, and their market is increasing continuously [14]. Microorganisms, including fungi and bacteria 15, naturally produce pectinolytic enzymes.

Pectinases have a crucial role in food industries. These enzymes are useful for fruit juice extraction and wine clarification; tea, cocoa, and coffee concentration and fermentation; vegetable oil extraction; preparation of jam and jellies and pickling[16]. Furthermore, these enzymes are used in paper and pulp industries, bleaching of paper, bio-sourcing of cotton, retting degumming of plant fibres, oil extraction, wastewater treatment, poultry feed additives, protoplast fusion technology, and bioenergy production [17].

Free-living bacteria and bacteria attached to marine sediments usually excrete large amounts of extracellular enzymes to hydrolyze intractable macromolecules [18]. So, in the present study, marine sediment samples are chosen for the emergent studies and production of novel enzyme pectinase. Marine sources of microbes are entirely different from other resources. Here we screened the isolated samples for pectinase activity and studied the morphological and biochemical characteristics. Further, gene sequencing and phylogenetic analysis were done for the selected bacterial strain.

MATERIALS AND METHODS:

Sample Collection: Marine sediment samples were collected from Kovilthottam, the coastal area of Puthenthura (Chavara), located in Kollam district, Kerala, India. The place was located at 3 km distance from the Neendakara fishing harbour area (South) and at 4 km distance from KMML chemical factory (North). The sediment samples were collected in sterile bottles and brought to the laboratory, stored at room temperature at 37°C until further analysis.

Isolation of Marine Bacteria: The collected marine sediment samples (1 ml) were serially diluted up to 10^{-9} with distilled water. Isolation of microbes was done by pour plate method [19-20]. The dilutions taken were 10^{-3}, 10^{-5}, 10^{-7} and 10^{-9}. The medium used to grow bacterial culture was zobell marine agar medium. The media was sterilized by autoclaving at 121 °C (15 lbs pressure) for 15 minutes. The plates were incubated at 37°C for 48 hrs. Isolated bacterial strains were streaked in zobell marine agar slant.

Screening for Pectinase Enzyme: All isolated bacteria were tested for pectinase production by using yeast extract pectin (YEP) medium. The pure culture colonies were picked up from each slant and streaked on YEP agar plates. The plates were incubated at 37 °C for 48 hours. After incubation, iodine-potassium iodide solution was added to detect clearance zone [21].

Morphological and Biochemical Characteristics: Gram staining, Motility, Indole production, Methyl red, Voges-proskauer, Citrate utilization, Nitrate reduction, Ureahydrlysis, Catalase, Oxidase, Casein hydrolysis were carried out. The potential bacterial strains were biochemically identified using Bergey's Manual of Determinative Bacteriology [22].

S-rRNA Sequence Analysis: The bacterial strain PE S7C is selected for 16S rRNA sequence analysis. The partial sequence of the 16S rRNA gene was amplified by polymerase chain reaction. Here explored the possibility of forwarding and reversed primers for amplification, 16S rRNA (27F & 1492R). DNA was isolated from the culture. Electrophoresed the DNA in 1% Agaroses and visualized it under UV. 16S region was PCR amplified with specific primers, and agarose gel visualization checked the amplicon for the appropriate site. The amplicon was gel purified using a commercial column-based purification kit (*In-vitrogen*, USA), and sequencing was performed with forward and reversed primers in ABI 3730 XL cycle sequencer.

Phylogenic Analysis: Sequence analysis was performed using the online tool BLAST of the NCBI database. Based on the maximum identity score E value, top most sequences were utilized for multiple sequence alignment (Clustal W2) and dendrogram was constructed. Forward and reverse sequences were assembled, and contig was generated after trimming the low-quality bases. The trimmed genetic sequences were then compared to

different 16S rRNA genes of different bacteria in the reference RNA sequence(16S rRNA) databases of the NCBI nucleotide BLAST website using BLASTIN 2.9.0+ Program to identify the genus of the selected isolate. The query sequence was constructed to FASTA format and was then used to create a phylogenetic tree.

RESULTS AND DISCUSSION:

Isolation and Screening of Pectinase-Producing Microorganisms: In the present study, marine sediment samples were collected, and the samples were serially diluted, pour plated, and incubated at 37^{o}C for 48h. About seven dominant morphologically distinct colonies were selected and streaked on the zobell marine agar slant. From this, five isolates were screened for pectinase-producing ability on YEP agar. The bacterial strains were PE S7C, PE S1B, PE S4A, PE S1C, and PE S1A. The zone formation around the bacterial growth was identified as the positive pectinase producer. Among the five isolates, two isolates (PE S7C & PES1B) showed maximum pectinolytic activity with a clear zone. In contrast, the other three isolates (PE S4A, PE S1C, and PE S1A) exhibited poor pectinolytic activity **Fig. 1, Table 1**. Therefore the efficient pectinase-producing isolates PE S7C were selected for further identification.

TABLE 1: PECTINASE ACTIVITIES OF VARIOUS STRAINS

Sl. no.	Bacterial Strains	Pectinase activity (Qualitative)
1	PE S7C	Positive
2	PE S1B	Positive
3	PE S4A	Negative
4	PE S1C	Negative
5	PE S1A	Negative

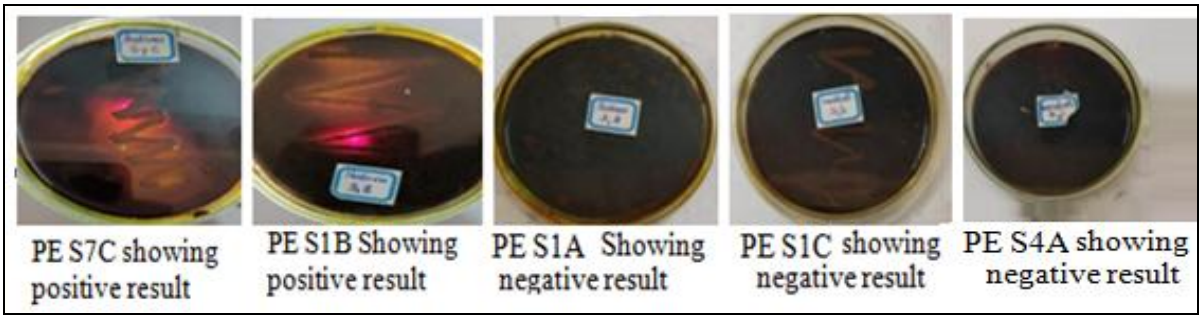

FIG. 1: SCREENING OF MICROORGANISMS FOR PECTINASE ACTIVITY BY USING YEP AGAR

Morphology and Biochemical Characteristics of Pectinase-Producing Bacterial Strains: Morphological and biochemical characterization of pectinase-producing bacterial strains PE S7C and PE S1B were performed in accordance with Berge's manual of determinative bacteriology.

TABLE 2: MORPHOLOGICAL AND BIOCHEMICAL CHARACTERISTICS OF PECTINASE-PRODUCING ISOLATES

Morphological characteristics	Bacterial strain	
	PES7C	PES1B
Gram's staining	+	+
Morphology	Rod	Rod
Motility	Motile	Motile
Biochemical test		
Indole (I)	Positive	Positive
Methyl red(MR)	Positive	Positive
Voges Proskauer (VP)	Positive	Positive
Citrate utilization	Positive	Positive
Nitrogen reduction	Positive	Positive
Urease test	Positive	Positive
Catalase	Negative	Negative
Oxidase	Negative	Negative
Casein hydrolysis	Negative	Negative

Gram's staining, motility, Indole, Methyl red, Voges Proskauer, Citrate utilization, Nitrate reduction, Urease, Catalase, Oxidase, and Casein hydrolysis were performed. The results are shown

in **Table 2. Table 2** describes that all bacterial strains morphologically appear to be a rod-shaped bacterium. After staining, the two isolates showed a purple (gram-positive) colour. All isolates showed positive for Indole, methyl red, VP, citrate, nitrogen reduction, and urease. In contrast, it was negative for catalase, oxidase, and casein hydrolysis.

Phylogenetic Analysis: 16S rRNA was PCR amplified with specific primers, and amplicon was checked for appropriate size by agarose gel visualization. The amplicon was gel purified using a commercial column-based purification kit (*In-vitrogen*, USA) and sequencing was performed with forward and reversed primers in AB1 3730 XL cycle Sequencer. The gel-purified product was sequenced. The 16S region was sequenced very well and was excellent for predicting the identity of organisms. Contig was obtained for 16S region after trimming off the low-quality bases, and good consensus was obtained. The phylogenetic tree, based on a comparison of the 16SrRNA sequences of pectinase-producing bacterial isolate PES7C and some of their close phylogenetic relatives, the tree was treated by the neighbor joining method. It revealed that the strain PES7C is *Bacillus cereus* **Fig. 2.**

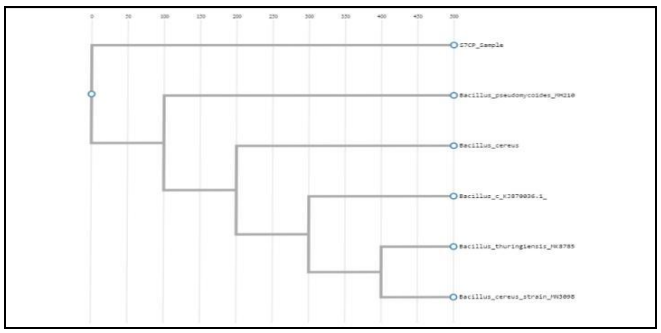

FIG. 2: PHYLOGENETIC TREE OF BACTERIAL STRAIN PE S7C

In this study, we isolated bacterial strains from marine sediment samples that produce industrially useful enzyme pectinase. Pectinases have crucial roles in food industries. These enzymes are useful for fruit juice extraction and wine clarification, tea, cocoa, and coffee concentration and fermentation, vegetable oil extraction, jam and jellies, and pickling preparation. Furthermore, these enzymes are used in paper and pulp industries, bleaching of paper, bio-scouring of cotton, retting, and degumming of plant fibers, oil extraction, wastewater treatment, poultry feed additives, protoplast fusion technology, and bioenergy production [23, 24]. The location of the marine sediment samples for the study showed the significance of the industrial area nearer to KMML factory and Neendakara harbour. *Bacillus* sp. produces a variety of compounds involved in the biocontrol of plant pathogens and promotion of plant growth, which makes them potential candidates for most agricultural and biotechnological applications [25].

CONCLUSION: In this study, we identified, isolated, and genetically characterized a pectinase-producing bacterial strain *Bacillus cereus* from marine sediment samples. The efficient pectinase-producing isolate PES7C was selected for gene sequencing and further identification. Morphological and biochemical characteristics indicated that the isolate was a gram-positive, rod-shaped, and motile organism. 16S rRNA sequence homology was compared, and a phylogenetic tree was constructed. This result confirmed the isolate PES7C, was *Bacillus cereus*. The present study disclosed that this pectinase-producing strain could also be helpful for industrial and clinical applications. The present study is a preliminary screening report of the diversity of *Bacillus sp* and their enzymes producing potential from marine sediments. It also revealed a high taxonomic diversity among these isolated *Bacillus*. Isolation of bacterial strains from marine sediment samples would also provide extensive scope to assess their biotechnological potential.

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