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Original Article

ISOLATION, SCREENING, AND CHARACTERIZATION OF CHITINASE PRODUCING BACTERIA FROM MARINE WASTES

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ABSTRACT

Objective: Aim of this study deals with screening and characterization of chitinase-producing bacteria from marine waste and its deposited soil along the coastal regions in Chennai.

Methods: The soil samples were collected aseptically and subjected to serial dilution to isolate the bacterial strains. Totally, 35 morphologically different microorganisms were isolated and were screened for their chitinolytic activity in colloidal chitin incorporated media through zone assay using Congo red stain. The biochemical tests were performed for the isolated to prove their validity and further with sequencing to determine the species.

Results: The isolates were screened based on the size of the zone formed. Best chitinase producers were subjected to biochemical tests and 16s ribosomal RNA sequencing. A novel strain, *Acinetobacter* ASK18, a gram-negative, motile organism was identified. Thus, the isolate may be a potent producer of chitinase, and the marine wastes can be utilized efficiently to generate a high value-added product.

Conclusion: A novel strain, *Acinetobacter* ASK18, would further be subjected to purification of the enzyme produced, and hence the active principle could be evaluated as an effective pharmacological drug in anticancer and antibacterial properties.

Keywords: Marine wastes, Chitinase, Congo red, Biochemical tests, 16s rRNA sequencing

INTRODUCTION

Chitin is considered as the second natural polymer next to cellulose with the structural unit of N-acetylglucosamine linked by β-1,4 bonds. It is present in the cell wall of higher fungi, exoskeletons of insect, and shells of crustaceans [1, 2]. Most of the seafood processing industries contain chitin in the processed marine wastes. These wastes may cause major environmental problems due to its easy deterioration. Such chitinous wastes are degraded through chemical processes like demineralization and deproteinization, which causes corrosive problems, low yield, and high costs [3]. Being more eco-friendly and cost effective method as compared to the chemical method for chitinous degradation, the enzymatic method can be adopted as an alternative. Chitinases (EC 3.2.1.14) are a set of enzymes that are produced by several bacteria, actinomycetes, fungi, and also by higher plants [4-8]. Chitinases play a major role in degrading the chitinous waste from the seafood industry and thus retains the carbon-nitrogen balance in the environment through the utilization of crustacean waste [9]. Shrimp waste is considered as a major source of chitin. The presence of chitinolytic microbes indicates the availability of chitin in the soil.

Chitinases also play a major role in many areas such as the production of single cell protein, growth factors [10, 11], mosquito control, a biocontrol agent of fungal pathogens, and isolation of fungal protoplasts [12, 13]. Thus, the need of microbial chitinase production has increased, and it serves two purposes: (i) reduce environmental hazards and (ii) increases production of industrially important value-added products.

Thus, the present study has been narrowed on isolation, screening, and characterization of chitinase producers from marine waste samples collected from Chennai.

MATERIALS AND METHODS

Chemicals

The materials, media, reagents used for this study were procured from Titan, SRL and Hi-Media, India.

Methods

Collection of soil samples

Prawn shell and depositing premises in the fish market of Tambaram and Vanagaram areas in Chennai, Tamil Nadu, India were selected for the soil collection. Soil at the depth of approximately 9–12 cm was collected in a sterile zip-lock cover with the help of a sterile spatula and placed in an ice pack for transportation to the laboratory and it was processed [14].

Preparation of colloidal chitin

To 10 g of chitin powder (Titan, India), added 120 ml of conc. HCl, incubated at 37 °C, 180 rpm for 1 h. The mixture was transferred through glass wool to 50% ethanol and thoroughly mixed to obtain a homogenous suspension. This was further transferred through filter paper and washed with distilled water until the colloidal chitin reaches pH 7. Colloidal chitin was collected and stored at 4 °C until use [15].

Isolation of chitinase producers

Chitin utilizing bacteria from the collected soil sample was isolated by serial dilution and spread plate technique. 1 ml of each dilution was plated in triplicates on nutrient agar medium supplemented with 1% colloidal chitin and incubated at room temperature (27 °C) for 3 d, and isolation of bacteria was carried out from the third day onward. The chitinase producers were selected based on the morphology, color, and growth in the colloidal chitin-incorporated medium [16].

Screening of chitinase-producing bacteria

Quadrant streak of all the isolates was carried out in nutrient agar plate supplemented with colloidal chitin to isolate the potential organism based on the chitinase produced.

Single streak inoculation measuring 2 cm length was performed for all the bacterial isolates on nutrient agar medium supplemented

with colloidal chitin and incubated at room temperature for 2 d. The plates were stained with 0.1% Congo red and distained with 1% NaCl, and the bacterial isolates producing a clear zone of more than 10 mm were selected. The screened pure isolates were stored in nutrient agar slants added with 1% colloidal chitin at 4 °C to maintain the viability of chitinase producers.

Characterization of bacterial isolates

Identification of chitinolytic bacterium

The isolates were identified through (i) their morphological and physiological properties according to Berger's manual of systematic bacteriology [17]. (ii) The nearly complete nucleotide sequence of 16s ribosomal RNA (rRNA) was determined using universal bacterial primers. The 16s rRNA sequence was compared to the sequences in the genbank nucleotide database by using Basic Local Alignment Search Tool (BLAST).

Isolation of genomic DNA for 16srRNA sequencing and polymerase chain reaction amplification

Total genomic DNA of all the isolates was extracted by phenol-chloroform method according to Sambrook *et al.* [18]. The concentration and purity of the DNA were estimated by agarose gel electrophoresis on ultraviolet (UV) transilluminator.

Molecular identification of the isolates was carried by 16s rRNA sequencing in which the 16s rRNA region was amplified with the universal forward and reverse primer: 8f (5-'AGAGTTT GATCCTGGCTCAG-3') and 1492r (5'-CGGTTACCTTGTTACGACTT-3') respectively using a gradient polymerase chain reaction (PCR) (Applied Biosystems, USA). The PCR amplification was performed with denaturation (95°C; 30 s), annealing (54 °C; 30 s), extension (72°C; 5 min) followed by a final extension (72 °C; 5 min). The PCR-amplified product was analyzed in 2% agarose gel added with ethidium bromide and 1 kb DNA ladder followed by UV trans-illuminator documentation. The PCR-amplified sample was sequenced with the same set of primers. Finally, a similarity search for the nucleotide sequence of 16s rRNA gene of the test isolate was carried out using a BLAST search at NCBI [19].

RESULTS AND DISCUSSION

Chitinase producing bacterial strains were isolated from the marine wastes (crustaceans waste and its deposited soil) from different places along the coastal areas of Chennai. Totally, 35 (labeled as ASK1–ASK35) different bacterial strains were isolated, of which 14 isolates were found to produce clear zone (>10 mm) when incubated in chitin-containing media and further stained with Congo red. Clear zone surrounding the colony indicates chitinase activity to break down chitin compound in medium [20]. The screened isolates were further narrowed down to choose the best producers of chitinase based on the zone formation (>20 mm). Five (ASK5, ASK18, ASK19, ASK22, and ASK31), best chitinase producers, were chosen and stained with Congo red (fig. 1).

These isolates were subjected to identification through biochemical tests and sequencing. Results of biochemical tests are tabulated as below (table 1). From this, it could be predicted that the isolates can be *Bacillus sp*., *Acinetobacter sp., Chitiniphilus sp.* The chitin degrading strains were identified at the species level by 16s rRNA sequencing. The result reveals that the identified strains (ASK5, ASK18, ASK19, ASK22, and ASK31) are *Chitiniphilus shianonensis*, *Acinetobacter sp., Bacillus badius, Acinetobacter venetianus*, and *Brevibacillus borstelensis,* respectively.

From the sequencing results, it is clear that the strain ASK18 belongs to *Acinetobacter* family. However, its species has not been identified and hence could be a novel strain. The strain, *Acinetobacter* ASK18, produced a zone of 43 mm. It is a Gram-negative, motile organism possessing positive results for oxidase and methyl red tests and negative for others.

Shrimp waste is the important source of chitin for commercial use. Bacteria produce several chitinases, to hydrolyze different form of chitin found in nature. Chitins can vary by the arrangement of NAG strands, the degree of deacetylation, and the presence of crosslinked structural components, such as proteins and glucans. Bacterial chitinases belong to family 18 of the glycosyl hydrolases [21, 22]. Bacterial chitinases have been classified into three groups (A, B, C) according to Watanabe *et al.* based on sequence similarities. From the screening of chitinase producers, it is revealed that the level of chitinase secretion varies with response to chitin induction [23].

Similarly from other reports it shows that like *Acetinibacter, Serratia marcescens* and different species of *Bacillus* has been reported to produce chitinase [24-26], and the result of the present study also confirms the same.

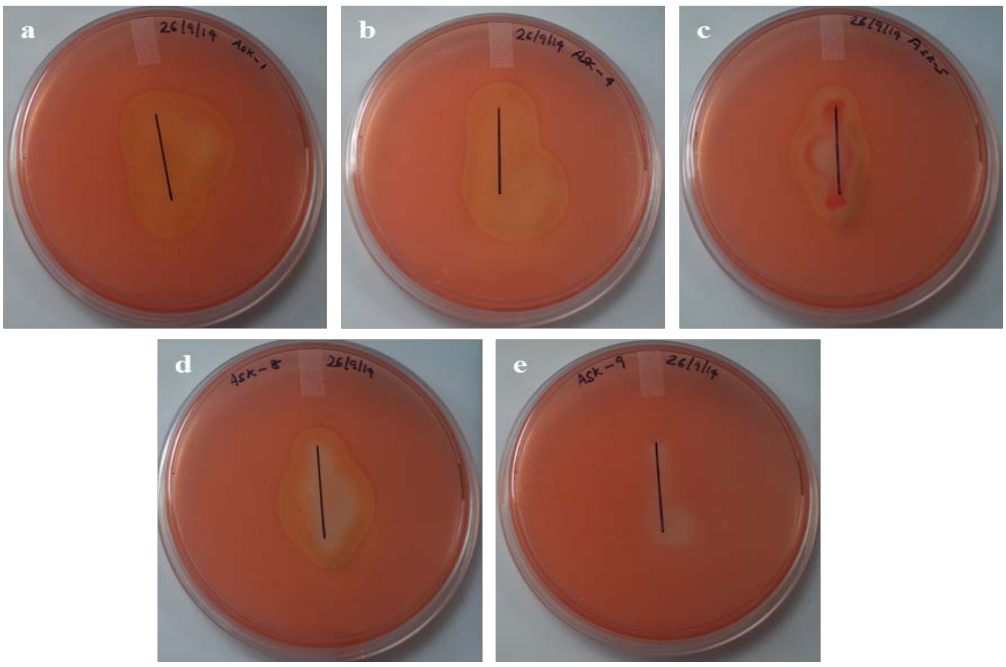

Fig. 1: Screening of isolates with Congo red stain—(a) ASK5, (b) ASK18, (c) ASK19, (d) ASK22 and (e) ASK31

Table 1: Biochemical characterization of screened isolates

Biochemical tests	ASK5	ASK18	ASK19	ASK22	ASK31
Gram's stain	Gram-negative	Gram-negative	Gram-positive	Gram-negative	Gram-positive
Starch Hydrolysis	Negative	Negative	Negative	Negative	Negative
Citrate Utilization	Negative	Negative	Negative	Positive	Negative
Indole	Negative	Negative	Negative	Negative	Negative
Oxidase	Positive	Positive	Negative	Negative	Positive
Catalase	Negative	Negative	Negative	Negative	Positive
Methyl red	Positive	Positive	Positive	Positive	Weak Positive
Voges Proskauer	Negative	Negative	Negative	Negative	Negative
Motility	Motile	Motile	Motile	Motile	Motile
Gelatin Hydrolysis	Negative	Negative	Positive	Negative	Negative

CONCLUSION

Chitin is a versatile and promising biopolymer with numerous industrial, medical, and commercial uses. It is estimated that nearly 10[11] tons of chitin is produced annually in the biosphere, much of it in the oceans. India has a potential of producing 10,000 tonnes of chitin per annum from the crustacean waste. At present, the chitin industry in India is utilizing only less than 20% of the shell waste with earnings of 100 crores annually. Thus, in the present study, the prawn shell waste is used as a source for the production of biomedically important enzymes (chitinase) and hence reduces the environmental hazards caused by shrimp shell decomposition.

CONFLICT OF INTERESTS

Declared none

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