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Biocontrol potential of Halotolerant bacterial chitinase from high yielding novel *Bacillus Pumilus MCB-7* autochthonous to mangrove ecosystem

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ABSTRACT

The multifaceted role of chitinase in medicine, agriculture, environmental remediation and various other industries greatly demands the isolation of high yielding chitinase producing microorganisms with improved properties. The current study aimed to investigate the isolation, characterization and biocontrol prospective of chitinase producing bacterial strains autochthonous to the extreme conditions of mangrove ecosystems. Among the 51 bacterial isolates screened, *Bacillus pumilus* MCB-7 with highest chitinase production potential was identified and confirmed by 16S rDNA typing. Chitinase enzyme of MCB-7 was purified; the chitin degradation was evaluated by SEM and LC-MS. Unlike previously reported *B.pumilus* isolates, MCB-7 exhibited highest chitinase activity of 3.36 U/mL, active even at high salt concentrations and temperature up to 60 °C. The crude as well as purified enzyme showed significant antimycotic activity against agricultural pathogens such as *Aspergillus flavus, Aspergillus niger, Aspergillus fumigatus, Ceratorhiza hydrophila* and *Fusarium oxysporum.* The enzyme also exhibited biopesticidal role against larvae of *Scirpophaga incertulas* (Walker). [Lep.: Pyralidae], a serious agricultural pest of rice. The high chitinolytic and antimycotic potential of MCB-7 increases the prospects of the isolate as an excellent biocontrol agent. To the best of our knowledge, this is the first report of high chitinase yielding *Bacillus pumilus* strain from mangrove ecosystem with a biocontrol role against phytopathogenic fungi and insect larval pests.

1. Introduction

Chitinases [EC 3.2.11.14] are enzymes that catalyze the hydrolysis of beta −1, 4-*N*-acetyl-glucosamine linkages in chitin polymers. Chitinases are produced by various bacteria such as *Aeromonas, Serratia, Vibrio, Streptomyces* and *Bacillus* and fungi such as *Aspergillus, Trichoderma* and *Penicillium*, plants, actinomycetes, arthropods and humans [1,2]. In plants, these enzymes mainly function in the defense against fungal and insect pathogens by destroying their chitin containing cell wall. Many bacteria and fungi containing the chitinolytic enzyme convert chitin into carbon and nitrogen that can serve as the energy source.

The antifungal activities of chitinase make it an attractive candidate for developing efficient biocontrol formulations for agriculturally important plants and demand an equally diverse array of chitin-modifying enzymes for specific needs [3]. Moreover, most of the degradation products of chitin are found to be biocompatible, nontoxic and biodegradable, increasing their application potential in various sectors of food and medicine, biotechnology, material and medical science, agriculture and environmental protection [4]. However, chitinases are not widely used in commercial scale due to their high cost of production, regardless of its high potentials in pest control and medicine preparation [5]. Thus, the search for better microbial sources of chitinase with profound biocontrol properties gain much relevance.

Among various ecological niches unique for their untapped microbial resources, mangrove forests stand out as a great hotspot with high biological diversity and activity contributing to novel bioactive compounds. The frequent variations in salinity and organic matter availability in mangroves often cause the autochthonous microbial flora to produce a wide array of chitinases. The current research article evaluated the antimycotic and anti insecticidal properties of *Bacillus pumilus* MCB-7, a noval mangrove bacterial strain.

2. Materials and methods

2.1. Sample collection

The bacterial strain *Bacillus pumilus* MCB-7 was isolated, screened and identified as per Rishad et al. [6]. A total of 30 different soil samples were aseptically collected from mangrove islands of Valanthakad backwaters of Cochin, Kerala, India; situated on the eastern side of the

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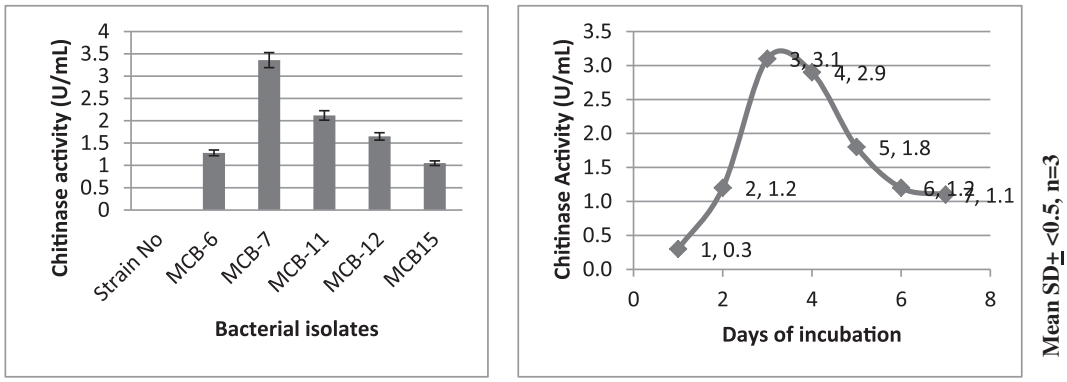

Fig. 1. Chitinase production by various isolates in colloidal chitin Broth [a] comparison of best 5 isolates [b] Chitinase production profile of selected isolate *Bacillus pumilus* MCB-7.

Vembanad ecosystem [9° 55′ 10. 24″ N latitude and 76° 20′ 01. 23″ E longitude].

2.2. Isolation of chitinolytic bacteria

Mangrove soil samples were enriched in a mineral medium containing colloidal chitin as sole carbon and energy source was used and incubated at 30 °C. Colloidal chitin was prepared by the method of Wiwat et al. [7]. Chitin agar medium amended with 0.5% colloidal chitin was used as a selective agar for the isolation of prominent chitinase producing bacteria after enrichment. The selected bacterial colonies showing clear zones [halo] on colloidal chitin agar were selected and grown in 100 mL of Luria-Bertani [LB] medium with 2% chitin powder at 30 °C for 72 h and the chitinase released into the medium was quantitatively measured.

2.3. Quantitative assays of chitinase activity

2.3.1. Colloidal chitin as substrate

The chitinase enzyme assay was carried out by the method of Miller et al. [8] as previously described. The released reducing sugar was determined relative to the *N*-acetyl-β-D-glucosamine standards from 100 to 1000 μg/mL concentration by the dinitro salicylic acid (DNSA) method. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmole of reducing sugar from colloidal chitin per minute. Protein concentration was measured against bovine serum albumin as standard according to Lowry et al. [9].

2.3.2. pNp-GlcNAc as substrate

The chitinase activity was quantified by measuring the release of p-nitrophenol from p-nitrophenyl-*N*-acetyl-β-D-glucosaminide. The assay mixture contained 0.4 mL of 25 mM sodium phosphate buffer (pH 7.0), 0.5 mL of 1 mM p-nitrophenyl-*N*-acetyl-β-D-glucosaminide and 0.1 mL enzyme solution. The assay mixture was then incubated at 37 °C for 10 min and the absorbance was measured at 410 nm. One unit of enzyme activity was defined as amount of enzyme required to release 1 μmole of p-nitrophenol per minute from p-nitrophenyl-*N*-acetyl-β-D-glucosaminide [10].

Table 1
Purification steps of chitinase from *Bacillus pumilus* MCB-7.

Purification steps	Total protein (mg)	Total enzyme activity (U)	Specific activity	Purification fold	Yield (%)
Crude extract	454	3360	7.40	1.00	100
Ammonium sulphate F80	235	2957	12.58	1.70	88.01
DEAE-cellulose column chromatography	48	1369	28.52	3.85	40.74
Biogel P-100	12	896	74.67	10.09	26.67

2.4. Bacterial identification

The genomic DNA of the selected 5 isolates were extracted using DNA extraction kit [Sigma Aldrich, Bengaluru, India] and identified by 16S rDNA typing. PCR reactions for 16S rDNA gene region amplifications were carried out in gradient thermal cycler [Agilent Sure cycler, USA] with 16S Forward Primer 8F: 5′-AGAGTTTGATCMTGG-3′ and 16S reverse primer 1492R: 5′-ACCTTGTTACGACTT-3′. The PCR products were further purified by QIAamp DNA Purification Kit [Qiagen] and sequenced at Eurofins Genomics Pvt. Ltd., Bengaluru, India. The sequences were analyzed using the BLAST [www.ncbi.nlm.nih.gov] search algorithm and was deposited in the NCBI Gene Bank database under appropriate accession numbers. The phylogenetic tree was constructed and evolutionary history was inferred using the Neighbor-Joining method by using MEGA 6.0 software.

2.5. Chitinase production by submerged fermentation

Chitinase production was performed with the promising isolate MCB-7 in a fermentation medium containing 1.0% colloidal chitin, 0.5% peptone, 0.5% yeast extract, 0.1% KH_2PO_4 and 0.01% $MgSO_4 \cdot 7H_2O$ at pH 7.0 and incubated at 37 °C in a shaker incubator at 150 rpm. 1% (v/v) inoculum consisting of 3×10^6 CFU/mL was used as the starter culture. After 72 h of incubation, the culture was centrifuged at 10,000 rpm for 10 min to obtain the crude enzyme extract. The supernatant obtained was further quantified for chitinase activity and protein content as described earlier.

2.6. Purification of chitinase enzyme

The crude chitinase enzyme extract was precipitated by ammonium sulphate and dialysed with 25 mM Na-phosphate buffer [pH − 7.4] at 4 °C overnight. Further purification of chitinase of *Bacillus pumilus* MCB-7 was carried out by the DEAE-cellulose column chromatography equilibrated with 25 mM sodium phosphate buffer and eluted with a linear gradient of 0 to 500 mM NaCl in the same buffer at a flow rate of 1.0 mL/min. The chitinase enzyme was further purified on Biogel P-100 column [Biorad, India]. All the steps were carried out at 4 °C. The different purified fractions were assayed for chitinase activity and SDS-Polyacrylamide gel electrophoresis. Subsequently, zymogram was demonstrated by copolymerizing 0.01% of glycol chitin [Sigma] in SDS-PAGE for the detection of chitinase activity [11].