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Original Article

Molecular Characterization and Antifungal Activity of Extracellular Chitinolytic Enzyme Producing *Paenibacillus elgii* TS33 isolated from Shrimp Shell Waste

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ABSTRACT

In this study an extracellular chitinase producing bacterium was isolated from shrimp shell waste. It produced circular, flat, smooth, opaque and white colonies on nutrient agar. These were facultative anaerobic, gram-variable rods with peritrichous flagella and formed ellipsoidal spores in swollen sporangia. The biochemical examinations revealed the positive reaction for test Methyl red, Triple Sugar Iron, Oxidase, Catalase, Urease, Starch hydrolysis, Casein hydrolysis, Glucose, Galactose, Maltose, Mannose, Mannitol, Xylose and negative reactions for the tests of Iodole, Voges-Proskauer, Citrate, Nitrate, Gelatin liquefaction, Arginine dihydrolation, Lysine decarboxylation, Ornithine decarboxylation, Arabinose. Molecular identification was performed by 16S rDNA amplification and sequencing. It was amplified using forward primer: 5′ TAG GGA AGA TAA TGA CGG 3′ and reverse primer: 5′ CCT CTA TCC TCT TTC CAA CC 3′. The amplified polymerase chain reaction product when run in agarose gel was found having a molecular weight approximately of ~1.4kp when compared with the DNA marker. The amplified product was sequenced and sequence confirmed the isolated bacterium was *Paenibacillus elgii.* Gene sequence was submitted to NCBI bearing Accession No. JQ735954. The isolated strain was named as *Paenibacillus elgii* TS33. Further, the extracellular chitinase enzyme was found to have a chitinolytic activity of 163U/ml in chitin substrate. *Paenibacillus elgii* TS33 showed antifungal activity against three fungal pathogens: *Fusarium solani, Aspergillus parasiticus* and *Aspergillus fumigates.*

Key Words: Chitin**,** Chitinase, Antifungal Activity, *Paenibacillus elgii.*

1. INTRODUCTION

Chitin is the second abundant polymer after cellulose, widely distributed in nature as the integument of insects, crustaceans, component of fungal cell wall and Entamoebal cyst wall [1]. The enormous amounts of chitin as well as

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chitosan continuously generated in nature require disposal and recycling on a formidable scale [2]. The catabolism of chitin typically occurs in two steps involving the initial cleavage of the chitin polymer by polysaccharides (Chitinase) into Chitin oligosaccharides, and then further cleavage to N-Acetyl glucosamine monomers by chitobiases [3]. Chitinases are a wide spread group of enzymatic systems that breakdown chitin an abundant insoluble linear polymer of β-1, 4-linked N-Acetyl glucosamine [4]. Chitinases are generally found in organisms such as bacteria, parasites, arthropods, crustaceans, higher plants and mammals and their properties are closely related to their biological functions [5,6,7]. Many chitinolytic bacteria show activity against the plant and animal fungal pathogens [8,9]. It is suggested that chitinolytic microorganisms have potential applications in the biocontrol of plant pathogenic fungi and insects as a target for biopesticides in many other biotechnological areas [10]. The oligosaccharides of chitin prepared by hydrolyzing with chitinase have various potential applications in the fields of food, agricultural and pharmaceutical industry. The bioconversion of chitinous materials has been proposed as a waste treatment alternative to the disposal of shellfish wastes [11]. Members of the genus *Paenibacillus* are facultative anaerobic, rod shaped, produce ellipsoidal spores in swollen sporangia. Some of these organisms excrete diverse assortments of polysaccharide hydrolysing enzymes and produce antibacterial compounds such as polymyxin, octopytin, baciphelacin, and an antifungal compound [12,13,14]. In this study chitinase producing *Paenibacillus elgii* was isolated from shrimp shell waste which showed antifungal activity against three fungal pathogens: *Fusarium solani*, *Aspergillus parasiticus* and *Aspergillus fumigates.*

2. METHODOLOGY

Preparation of Colloidal Chitin

The colloidal chitin was prepared following Robert and Selitrennikoff [15] method with slight modification by weighing the 20gms of chitin powder which were slowly dissolved into 350ml of concentrated hydrochloride acid then left at 4^0C overnight incubation on stirring position. The mixture was added to the 95% ice cold ethanol with rapid stirring and kept overnight at 35 °C. The precipitate was collected by centrifugation at 5000g for 20 minutes at 4°C. The precipitate was washed with sterile distilled water until the colloidal chitin become neutral (pH 7.0).

Chitinolytic Activity

A total of 10 shrimp shell waste samples were collected for the isolation of chitin degrading bacteria. The samples were collected a sterilized in zip lock bag and brought to the laboratory. One gram of sample was weighed and transferred to a vial containing10 ml of sterile water, votexed to dislodge bacterial cells from the sample particles. These were then allowed to settle down for 30 min. The supernatant was diluted10-fold and 0.1 ml (10^{-5}, 10^{-6}, 10^{-7} dilutions) was spread on the nutrient agar medium supplemented with 0.1% (w/v) chitin (pH 7.5). These plates were incubated at 37 °C for 24 hrs. Bacterial colony with highest zone of clearance was selected, subcultured, purified by quadrant streaking. It was named as TS33 and stored on nutrient agar slants at 4°C [16].

Identification of Chitinolytic bacterium TS33

The morphological and biochemical characterization of the isolated bacterial strain was performed according to method of Gordon et al., [17] and using API 20E strips.

Microscopic morphology

The microscopic identification of bacterial strain TS33 was done by gram staining, capsule staining, endospore, flagella staining.

Biochemical Tests

the biochemical identification of bacterial strain TS33 was done *viz* iodole, methyl red, voges-proskauer, citrate, triple sugar iron, oxidase, catalase, urease, nitrate, urease, gelatin liquefaction, starch hydrolysis, casein hydrolysis, arginine dihydrolation, lysine decarboxylation, ornithine decarboxylation, glucose, galactose, maltose, arabinose, mannose, mannitol and xylose carbohydrate fermentation tests.

Molecular characterizations of the chitinolytic bacterial isolate TS33

Genomic DNA was extracted from chitinolytic bacterial isolate TS33 using HiPurATM Bacterial and Yeast Genomic DNA Purification Spin Kit (Himedia). To determine the sequence of the 16S rRNA gene, a DNA fragment of ~ 1.4 kb was amplified by PCR from the genomic DNA of the sample using universal eubacteria-specific primers: 27F (5-AGAGTTTGATCMTGGCTCAG-3) and 1492R (5-GGYTACCTTG TTACGACTT-3), bacterial isolate TS33 was mixed with a solution containing each primer at a concentration of 1 µM, 0.25 mM dNTPs and 2.5 units of Taq polymerase (Promega) in PCR buffer with 2.0 mM MgCl . PCR amplification was performed in an Eppendorf thermal cycler using the program: a 5 min denaturation step at 94°C, 30 amplification cycles of 1 min at 94°C, 1 min at 56°C and 1 min at 72°C, with a final extension step of 10 min at 72°C. The amplified PCR products were purified using GeneJET PCR purification kit (Fermentas life science) and sequenced by automated sequencer (ABI 3730xl Genetic) at Xcelris Labs Ltd. The forward and reverse sequences were edited using Bioedit program [18]. The sequence of obtained from chitinolytic bacterial strain TS33 was compared to 16S rRNA gene sequences available in the databases of the National Center for Biotechnology Information by BLASTN homology search [19]. Phylogenetic analysis included the 16S rRNA gene sequences of the local isolate TS33 and the reference strains obtained from GenBank. The evolutionary history was inferred using the Neighbor-Joining method [20]. The evolutionary distances were computed using the Kimura 2-parameter method [21] and are in the units of the number of base substitutions per site. Phylogenetic and Evolutionary

analyses were conducted in MEGA7 [22]. Sequences were submitted in Gene Bank and accession numbers were obtained

Chitinase Assay

The chitinase assay was done according to Imoto and Yagashitha [23] using colloidal chitin as the substrate. The enzyme solution (0.2ml) was added to 1ml substrate solution, which contained purified 0.3% colloidal chitin in sodium phosphate buffer (50mM, pH 6.0). The mixture was incubated for three hour at 55°C then centrifuged at 10000g for 10 min. After centrifugation the reaction was stopped by boiling the 450µl of supernatant, 650µl of 0.5M sodium carbonate and 0.05% potassium ferricyanide reagent for 15 minutes. The reducing sugar level were determined relative by keeping (10-200µl/ml) N-Acetyl- -D glucosamine as standard [24]. The substrate as well as enzyme blanks were prepared and the absorbance was measured at 420nm in ultra violet visible spectrophotometer. One unit of chitinase activity is defined as the amount of enzyme producing 1µmol reducing sugar per minute. The formula applied to calculate the enzyme activity was used as Enzyme Activity (U/ml) is equal to the change in absorbance per minute x 1000 x total volume of reaction mixture divided by Molar extinction co-efficient x Volume of enzyme in Assay

Analyzing Antifungal Activity

The spot inoculation method was followed for antifungal activity of bacterial strain *Paenibacillus elgii* TS33 [25]. The nutrient agar medium was prepared, sterilized at 121°C, poured into the sterilized peteri plates and allowed to solidify under aseptic conditions. After solidification bacterial strain *Paenibacillus elgii* TS33 was spot inoculated on nutrient agar medium and incubated at 37^0C for 48 hours. After incubation the molten potato dextrose agar medium containing the spores of test fungus, was spread on the same plate and reincubated at 27^0 C for 3 days.

3. RESULT

Analysis of Chitinolytic Activity

After the incubation the bacterial strain which showed a highest zone of clearance in the colloidal chitin medium indicated that it had the ability to produce extracellular chitinase which utilizes the chitin as sole source of nitrogen and leads to the formation of zone around the colonies. It was named as TS33 and selected for further analysis.

Identification of Chitinolytic Activity TS33

The isolated bacterial strain TS33 under microscope showed rod shaped gram positive bacilli with centrally located oval shaped spore, capsulated, flagellated bacteria.

Biochemical Tests

The isolated bacterial strain TS33 was biochemically identified as *Paenibacillus* based on interpretations of various reaction which are represented in Table-1

Confirmation by 16S rDNA gene

The amplified PCR product of TS33 when run in agarose gel electrophoresis, gave a DNA band approximately of ~ 1400 bp when compared with the DNA marker. Sequencing of PCR product revealed presence of 1401bp. The blast analysis (NCBI) showed it is 99% similar with *Paenibacillus elgii*. Thus the isolated bacterial strain was confirmed as *Paenibacillus elgii* and was named as *Paenibacillus elgii* TS33. Sequence was deposited in GenBank under accession number **(**JQ735954**).**

Phylogenetic Analysis

The evolutionary history was inferred in the Neighbor-Joining method and optimal tree with the sum of branch length = 0.04468882 is shown Figure 1. The isolated strain TS33 was found closely related to *Paenibacillus elgii* strain NBRC 100335.

Chitinase Assay

The chitinase enzyme of *Paenibacillus elgii* TS33 showed 163U/ml of chitinolytic activity.

Antifungal Activity of *Paenibacillus elgii* TS33

The antifungal activity was performed against *Fusarium solani*, *Aspergillus parasiticus* and *Aspergillus fumigates* in which *Paenibacillus elgii* TS33 showed the maximum zone of inhibition against *Fusarium solani* which was 17mm followed by *Aspergillus parasiticus* (15mm) and *Aspergillus fumigates* (14mm) respectively.

4. DISCUSSION

Chitinases play an important role in the breakdown of chitin and potentially in the utilization of chitin as a renewable resource. Screening is one of the most efficient and successful ways of searching for new or suitable microbial enzymes [16]. The influence of the carbon sources on chitinase production demonstrated that monosaccharides inhibited chitinase biosynthesis. Recently, interest in the *Paenibacillus genus* has been rapidly growing, for many *Paenibacillus* species have been shown to produce various enzymes and antimicrobial substances, such as xylanase and polymyxins [26,14]. There are more than 110 species that have been discovered in genus and *Paenibacillus elgii* strain B69 was isolated from soil samples collected from Tianmu Mountain in Hangzhou, China, and displays significant activity against all tested microorganisms [27]. In our study we have isolated a bacterium showing a clear zone in chitin media from the shrimp shell waste and it was identified morphologically, biochemically and molecularly as *Paenibacillus elgii*. It indicated the bacteria produced the chitinase enzyme and degrade the chitin in this media, for utilizing the carbon element [28]. Chitin is a virtuous source of carbon and it is essential for the bacterial growth [29,7]. Then assay of chitinase enzyme showed the 163U/ml of chitinolytic activity in colloidal chitin media substrate. One unit of chitinase activity is defined as the amount of enzyme producing 1µl mol reducing sugar per minute [30]. Biological control of soil-borne diseases has been a major alternative to chemical fungicides, and many bacterial biocontrol agents have been found to inhibit the turf pathogens [31]. Of these bacteria, endospore-formers including Bacillus and Paenibacillus spp.

are generally known to have advantages of prevalent presence in soils, tolerance to thermal stress, rapid growth in liquid fermentation, feasibility for formulation, and high survival rate for long period during storage. *Paenibacillus elgii* TS33 showed antifungal activity against the three fungal pathogens which are *Fusarium solani, Aspergillus parasiticus, Aspergillus fumigates.* The zone of inhibition indicated the antifungal activity and the bigger zone indicated the strong activity. *Paenibacillus elgii* TS33 showed the maximum zone of inhibition against *Fusarium solani* which is more than 15mm. But this bacteria was effectively maintained the antifungal activity for a long time in *Aspergillus parasiticus, Aspergillus fumigates* [32]. The genus *Paenibacillus* was discovered and proposed by Ash et.al [33]. The antimicrobial activity of *Paenibacillus elgii* found against the fungal and bacterial species [34]. The potential antagonistic activity of Paenibacillus *polymyxa* act against the different mycotoxigenic fungal strains [35]. The broad antimicrobial spectrum and production of different antibiotics by the same species or even by one single strain has already been described in different bacterial genera, including Paenibacillus [36, 37].

Table 1: The biochemical test reactions of bacterial strain TS33 isolated from shrimp shell waste

Test	Reaction Result	Test	Reaction Result
Indole	Negative	Casein Hydrolysis	Positive
Methyl Red	Positive	Arginine dihydrolation	Negative
Voge us Proskauer	Negative	Lysine decarboxylation	Negative
Citrate	Negative	Ornithine decarboxylation	Negative
Triple Sugar Iron	Positive	Galactose	Ferment A^+
Oxidase	Positive	Glucose	Ferment A^+
Nitrate	Negative	Maltose	Ferment A^+
Catalase	Positive	Arabinose	Negative
Urease	Positive	Mannose	Ferment A^+
Gelatin Hydrolysis	Negative	Mannitol	Ferment A^+
Starch Hydrolysis	Positive	Xylose	Ferment A^+
Identified = ***Paenibacillus elqii***			

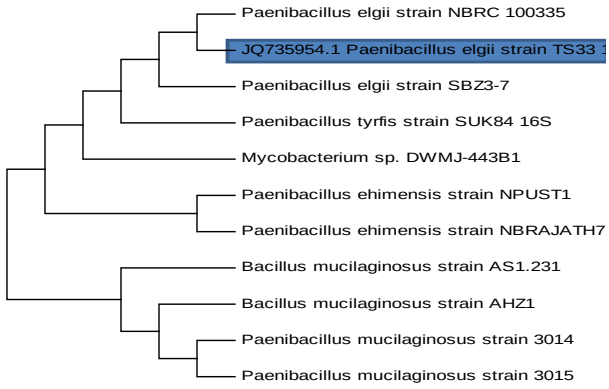

Fig 1: The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.04468882 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The analysis involved 11 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1319 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

5. CONCLUSION

In this research work we have isolated a chitinolytic bacterial strain which was identified morphologically, biochemically and on molecular basis as *Paenibacillus elgii* TS33. The *Paenibacillus elgii* TS33 produces the extracellular chitinase and showed 163U/ml of chitinolytic activity. *Paenibacillus elgii* TS33 showed the maximum zone of inhibition against *Fusarium solani* followed by *Aspergillus parasiticus* and *Aspergillus fumigates* respectively. Thus our study suggests that *Paenibacillus elgii* TS33 can be used as biocontrol against fungal pathogens.

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