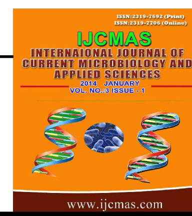

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Original Research Article

Chitinase production from marine wastes by *Aspergillus terreus* and its application in degradation studies

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A B S T R A C T

Chitinolytic enzyme producing *Aspergillus* species have long been recognized as an agent for biowaste management. Chitin is the main structural component of fungi. Chitinase is an enzyme responsible to metabolize the chitin. *Aspergillus terreus* CBNRKR KF529976*,* isolated from the marine soils of Pichavaram, Tamil Nadu was used successfully for the biodegradation of four different marine wastes-Crab shell, Snail shell, Shrimp shell, Fish scales in favor of the production of highly active chitinase enzyme. The crude chitinase was characterized and maximum activity was obtained in reaction mixture of 50°C incubation temperature, 2 ml crude enzyme, 0.5 ml of 10% colloidal chitin, pH 6 and reaction time of 10 min. Maximum enzyme activity was obtained in the case of Shrimp wastes (4.7 U/min), followed by Snail shell (4.3 U/min), Crab shell (4.2 U/min) and fish scales (3.7 U/min). The amount of marine wastes degraded were as follows-Crab shell (0.24g), Snail shell (0.11g), Shrimp shell (0.46g) and Fish scales (1.49g).The study is of considerable significance in the case of bioremediation of marine wastes.

Keywords

Aspergillus terreus; Chitinases; marine wastes; Pichavaram; Bioremediation

Introduction

Chitinase is the second most abundant polysaccharide in nature after cellulose, and it largely exists in wastes from processing of marine food products (crab, shrimp and krill shells as well as fish scales). About 10^{11} ton of chitin is produced annually in the aquatic biosphere alone (Wang and Chio, 1998). The waste generated from the worldwide production and processing of shell-fish and fish scales is a serious problem of growing magnitude. This abundant waste may pose environmental hazard due to the easy deterioration (Mejia-Saules *et al*., 2006). Chitinases (EC 3.2.1.14) are glycosyl hydrolases which catalyze the degradation of chitin. These enzymes have a wide range of biotechnological applications such as preparation of pharmaceutically important chitooligosaccarides and N-

acetyl-D-glucosamine (Kuk *et al*., 2005; Pichyangkura *et al*., 2002; Sorbotten *et al*., 2005), isolation of protoplasts from fungi and yeasts (Dahiya et al., 2005), preparation of single-cell protein (Vyas and Deshpande, 1991), control of pathogenic fungi (Mathivanan *et al*., 1998) and treatment of chitinous waste (Wang and Hwang, 2001). Chitinases are present in a wide range of organisms including bacteria, fungi, insects, higher plants and animals, and play important physiological and ecological roles (Cody *et al*., 1990; Duo-Chuan, 2006). The objective of the present study was to isolate the fungus *Aspergillus terreus* CBNRKR KF529976 from marine soils and characterize its growth conditions for maximum biodegradation of marine waste in favor of the production of highly active chitinase.

Materials and Methods

Isolation and characterization of Marine fungus

Sediment samples were collected from various stations of Pichavaram mangrove ecosystem (Lat, 11º 27'N, Long.79º 47' E) situated along the southeast coast of India. Sediment samples were collected from the rhizosphere of the mangrove plants. The soil samples were serially diluted by serial dilution method. 0.1ml from 10^{-3}, 10^{-4} and 10^{-5} dilution tube were transferred to PDA media and spread over the entire surface of the media using spreader and incubated for a week at 27°C.

Isolation of genomic DNA and 18S rRNA sequencing

Genomic DNA Isolation

The genomic DNA was isolated by transferring 1.5 ml of the culture to a micro centrifuge tube and centrifuged for 2 min. Then the supernatant was discarded and the pellet was re suspended in 467µl TE buffer by repeated pipetting, then 30µl of 10% SDS and 3µl of 20 mg/ml proteinase K were added and incubated for 1 hr at 37°C. After incubation an equal volume of chloroform was added and mixed well by inverting the tube until the phases are completely mixed. Carefully the DNA/phenol mixture was transferred into a fresh tube and centrifuged for 2 min. The upper aqueous phase was transferred in to a new tube. An equal volume of chloroform was added again mixed well and transfer to a new tube and centrifuged for 2 min. The upper aqueous phase was transferred to a new tube. Then 1/10 volume of sodium acetate and 0.6 volumes of isopropanol was added and mixed gently until the DNA precipitates. The DNA was spooled onto a glass rod (or Pasteur pipette with a heat-sealed end). The DNA was washed by dipping the end of rod into 1 ml of 70% ethanol for 30 sec. The DNA was re suspended in 100-200µl TE buffer.

PCR amplification of 18S rRNA

PCR amplification of 18S rRNA gene, from the purified genomic DNA was carried out using the universal fungal primer set, (Forward Primer) 5'-GACTCAACACGGGGAAACT-3' and (Reverse primer) 5'-AGAAA GGAGG TGATC CAGCC-3'.The reaction conditions were as follows: initial denaturation at 94 °C for 4 min, 40 amplification cycles of denaturation at 94°C for 1 min, annealing at 48 °C for 1 min and primer extension at 72 °C for 3 min; followed by a final extension at 72 °C for 10 min. Aliquots of the PCR products (5 µl) were analyzed in 1% (w/v) agarose gels by horizontal gel electrophoresis. DNAs were visualized by

UV excitation after staining with ethidium bromide (0.5 mg/L).

PCR product purification

The unpurified DNA sample was dissolved (at least 10-15µl) in 50µl of PCR cleanup solution and incubate at 55 ºC for 15-20 minutes. The mixture was Centrifuge at 12000 rpm for 15 minutes, during which time the contaminants was released into the supernatant and the supernatant was discarded at the end of the centrifugation. Further the DNA was precipitated by the addition of 600µl of 80% ethanol and centrifugation at the same conditions as before. The residual cleanup solution and the contaminants were removed along with ethanol by discarding the supernatant. Finally, the DNA pellet was dried and dissolved in 10-15µl of Milli Q water.

Sequencing

The sequencing of the target gene was done using ABI-Big Dye Termintor v3.1 Cycle Sequencing Kit using ABI 3100 automated sequencer by National Fungal Culture Collection of India (NFCCI), Pune, India. The tube was placed in the thermal cycler. The thermo cycler was programmed as follows: 25 cycles of [96° C for 10 sec, 50° C for 5-10 sec, 60^{0} C for 4 min] then ramp to 4^{0} C.

Purification of sequencing extension product by isopropanol precipitation method

The tube was spin and transferred by pipetting entire sequencing reactions into 1.5 ml micro centrifuge tube. Then 40 ml of 75% isopropanol, or 10 ml of de ionized water and 30 ml of 100% isopropanol was added and mixed by vortexing and left at room temperature for >15 min to precipitate products. The tube was centrifuged for a minimum of 20 min at maximum speed in a micro centrifuge. The supernatant was aspirated completely with a separate pipette tip for each sample, being careful not to disturb the DNA pellet, and then it is discarded. About 125 to 250 ml of 75% isopropanol was added to the tube and vortex briefly and centrifuged for 5 min at maximum speed, and the supernatant was aspirated as in above step. The sample was dried for 10 - 15 minutes and stored at -200 C until ready for electrophoresis. The purified extension products were separated in the ABI 3730xl DNA Analyzer by Capillary Electrophoresis.

The analysis of nucleotide sequence was done in Blast-n site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST). The alignment of the sequences was done by using CLUSTALW (www.ebi.ac.uk/clustalw).

Phylogenetic analysis

A phylogentic analysis of the isolate was performed to determine how the 18S rRNA sequence of the isolate and related strain might have been derived during evolution. The evolutionary relationships among the sequences were depicted by placing them as outer branches on a phylogenetic tree. The branching relationships on the inner part of the tree reflect the degree to which different sequences are related. Sequences that were very much alike were located as neighboring outside branches and joined to a common branch beneath them. The objective of phylogenetic analysis is to find out all of the branching relationships in the tree along with branch lengths. For this phylogenetic tree was constructed using the aligned sequences by the neighbor joining method using Kimura-2-

parameter distances in MEGA5 software. Distances between the studied sequences helps in understanding the evolutionary distances among the species.

Criteria for species identification

Identification of species through sequence similarity basis was performed according to criteria used by Bosshard *et.al* which states the following selection parameters: (a) when the percentage similarity of the query sequence and the reference sequence is 99% or above, the unknown isolate would be assigned to reference species; (b) when percentage similarity is between 95 – 99 %, the unknown isolate would be assigned to the corresponding genus; (c) when percentage similarity is less than 95 %, the unknown isolate would be assigned to a family.

Chitinase production and Biodegradation studies

Marine wastes

Four different kinds of marine wastes were used for the chitinase production. Fish scale [*Catla* (Bengal Carp)], Crab shell (*Scylla serrata)* and Snail shell (*Conus ebraeus)* waste were collected from the fish market in Perundurai, Tamil Nadu. Shrimp shell [*Fenneropenaeus indicus* (formerly *Penaeus indicus*)] waste was imported from the fish market in Mumbai. The scales waste was washed with tap water then distilled water. Thereafter, the wastes were exposed to water vapor and air dried at room temperature (Wang et al., 2005, 2006)

Inoculum and cultivation

A.terreus was maintained on PDA agar slants, where the fungus was grown for 5 days at 30 ± 2°C. The stocks were kept in the refrigerator and subcultured at monthly intervals. Spores suspension of A. terreus was prepared by washing 5 days old culture slants with sterilized saline solution (0.9% NaCl) and shaking vigorously for 1 min. Spores were counted by a haemocytometer to adjust the count to approximately 14.6 x 10^6spores/ml. The organism was allowed to grow in 100 ml aliquots of mineral salt medium of the following composition(g/l): waste, 20; $(NH_4)_2SO4$, 2; K_2HPO4, 1; $MgSO_4.7H_2O$, 0.5; KCl, 0.5; NaCl, 5; $CaCl_2$, 0.02; $FeSO_4.7H_2O$, traces and pH-6 (Al-Nusaire, 2007) and dispensed in 250 ml Erlenmyer flasks. Standard inocula (14.6 x 10^6spores/ml) were used to inoculate the flasks which were then incubated at 30 ± 2°C under shaking conditions (otherwise stated) at 150 rpm for 5 days. Thereafter, biomass was picked up, washed thoroughly to determine the dry weight at 80°C for constant weight. The residual fermentation products were centrifuged at 5000 rpm for 15 min in a cooling centrifuge. The clear supernatant was used to determine extracellular protein and considered as crude enzyme to assay chitinase activity (Rattanakit et al., 2007).

Analytical methods

Total protein assay

The extracellular protein was determined calorimetrically using Biuret methods.

Chitinase activity assay

Chitinase activity was measured using colloidal chitin as substrate (Bindo et al., 2005). Enzyme solution (0.5 ml) was added to 0.5 ml of substrate solution, which contained 10% colloidal chitin in phosphate buffer (0.05 M, pH 5.2) and 1 ml distilled water. The mixture was then incubated in shaking water bath at 50°C for 10 min, thereafter 3 ml of 3; 5-

dinitrosalicylic acid reagent was added. The mixture was placed in a boiling water bath for 5 min, after cooling, the developed color, as indication to the quantity of released N-acetylglucosamine (NAGA), was measured spectrophotometrically at 575 nm. The amount of NAGA was calculated from standard curve of NAGA. Chitinase activity (u/min) = the amount of enzyme releasing 1 μmol NAGA per min from colloidal chitin, under the assay conditions (Bindo et al., 2005).

Specific activity = Chitinase activity (U/min) / Extracellular protein

Results and Discussion

Morphological identification of the fungal isolates obtained from the soil sample

The isolated fungi were purified by repeated sub-culturing on the Potato Dextrose Agar medium at regular intervals and incubating at 29°C. The isolates were identified based on the colony morphology, microscopic observation and molecular identification (St-Germain et al., 1996; Collier et al., 1998).

Systematic position of *Aspergillus*
Kingdom: Fungi.
Phylum: Ascomycota
Order: Eurotiales
Family: Trichocomaceae
Genus: Aspergillus,
Species: terreus
Sub-species-CBNRKR KF529976

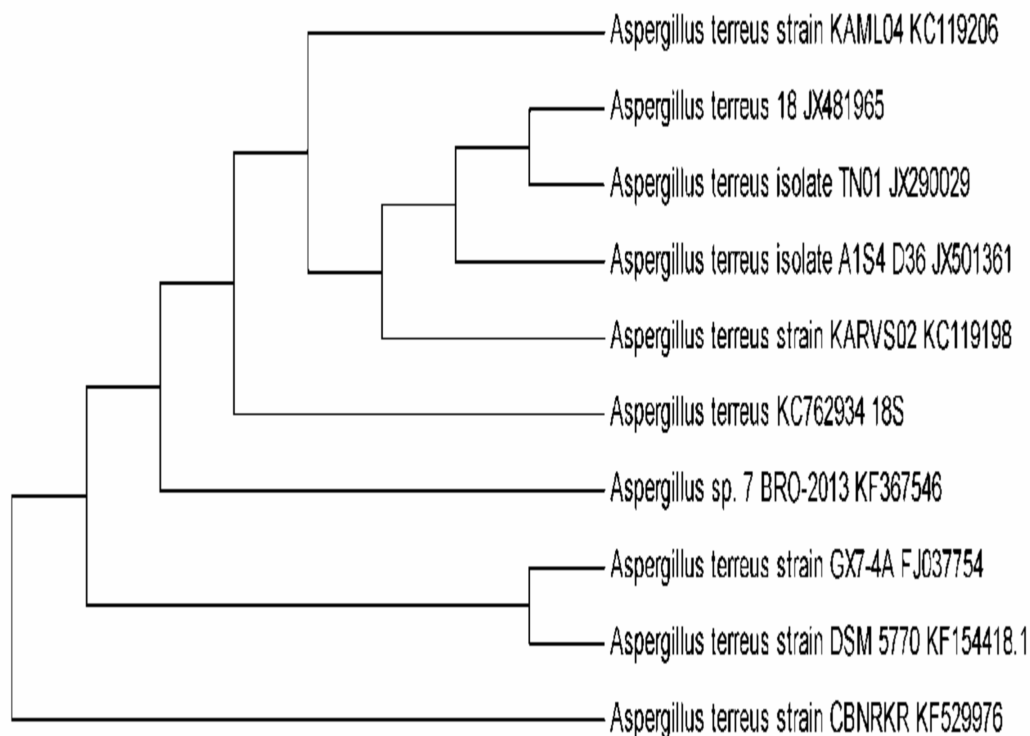
Identification and phylogenetic position of fungal isolates

The identification was done based on 18S rRNA gene sequencing. The 18S rRNA sequences of the isolates were compared with the data present in NCBI. The BLASTn of the isolates was showing 100% homology with *Aspergillus* spp. The sequence was submitted to the Gene Bank under the accession number KF529976. To analyze the phylogenetic position of the 18S rRNA sequence. The phylogenetic tree was constructed using Mega5 by neighbor-joining tree using Kimura-2-parameter with 1000 bootstrap replication. The phylogentic relation was determined Fig. (2).Shows the phylogenetic relationship between the isolates and other related fungi. The homology assay result indicated the isolates were in the phylogenetic branch of *Aspergillus* spp.

Chitinase production from various marine wastes

The fungus was allowed to grow under shaked conditions (150 rpm) for 5 days (120 h) on aliquots of 100 ml medium dispensed in 250 ml Erlenmeyer flasks. The first 5 days (120 h) represent the logarithmic growth phase of *A. terreus*. The highest chitinase activity recorded after 120 h (5 days) of growth was found to be 4.7 U/min in the case of Shrimp shell and it was concomitant with extracellular protein (about 1.2 mg/100 ml) produced by the fungus. The chitinase activity was found to be 4.3 U/min in the case of Snail shell waste while its extracellular content was found to be 0.5mg/100 ml. Crab shell waste showed chitinolytic activity of 4.2 U/min and its extracellular protein content was 0.9 mg/100ml.The lowest chitinase activity was observed in the case of fish scales waste (3.7 U/min) and its extracellular content was 2 mg/100ml. In accordance with these findings it was reported by Ghanem et al. (2010) that the maximum chitinase activity is 4.309 U/min in case of chitinase production from fish scales wastes from *A.terreus*.

Figure.1 Phylogenetic tree of *Aspergillus terreus* CBNRKR KF529976 showing homology with *Aspergillus* spp.



Similar results were also obtained in the case of chitinase production (3.86 U/min) from shrimp shell waste using *A.alternata.* (Ghanem et al., 2011)

Biodegradation studies

To study the effect of the chitinolytic enzyme activity on the different types of substrates, 2g of marine wastes was added to 100 ml of the medium in 250 ml Erlenmyer flasks. The amount of biomass left after 5 days incubation was as follows- Crab shell (0.24g), Snail shell (0.11g), Shrimp shell (0.46g), Fish scale (1.49g). Rattanakit et al. (2003) reported that as the size of fish scales waste increase, chitinase production by *Aspergillus* sp. decreases. It was indicated Ghanem et al. (2010) that larger sizes of fish scales were more convenient for higher chitinase production by A*spergillus* spp. than finest scales. This due to the differences between constituents and architecture of fish scales and shrimp shellfish wastes and also test organisms.

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