



Chitinase production from Seafood wastes by Plant pathogen *Bionectria CBNR BKRR* sps and its application in Bioremediation studies

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Abstract

Bionectria CBNR BKRR, isolated from the marine soils of Pichavaram, Tamil Nadu was used successfully for the biodegradation of four different sea food wastes-Crab shell, Snail shell, Shrimp shell, Fish scales to produce highly active chitinase enzyme. The crude chitinase so extracted was characterized and maximum activity was calculated from the reaction mixture containing, 2 ml crude enzyme, 0.5 ml of 10% colloidal chitin, pH 6 and a reaction time of 10 min at 50°C incubation temperature. Maximum chitinase enzyme activity was obtained in the case of fish scales (5.8 U/min) Shrimp wastes (4.0 U/min), followed by Snail shell (3.9 U/min), Crab shell (3.7 U/min). The amount of seafood wastes degraded was as follows-Crab shell (0.27g), Snail shell (0.01g), Shrimp shell (0.42g) and Fish scales (1.64g). The results indicated that *Bionectria CBNR BKRR* is efficient fungus to produce highly active chitinase when grown in statistically optimized medium containing Snail shell, Crab shell, Fish scales and Shrimp shells as substrates.

Keywords: *Bionectria CBNR BKRR*, Chitinases, marine wastes, Pichavaram, Bioremediation.

Introduction

Chitin is the second most abundant renewable carbohydrate polymer in nature after cellulose and possibly the most abundant in marine environments¹. It largely exists in wastes from processing of marine food products (20 to 58% of its dry weight)². The waste generated from the world-wide production and processing of shellfish is a serious problem of growing magnitude. This abundant waste may pose environmental hazard due to the easy deterioration³.

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 chito-oligosaccharides and N-acetyl-D-glucosamine⁴, isolation of protoplasts from fungi and yeasts⁵, production of single-cell protein⁶, control of pathogenic fungi⁷ and treatment of chitinous waste⁸. A wide range of microorganisms have the ability to degrade chitin by producing chitinases for nutrition, antagonism and combating parasites⁹. Chitinases are produced by several fungal species¹⁰.

The objective of the present study was to isolate the fungus *Bionectria CBNR BKRR* from marine soils and characterize its growth conditions for maximum bioremediation of seafood industry wastes to produce highly active chitinase.

Material and Methods

Isolation and characterization of Marine fungus, Isolation and screening: Sediment samples were collected from various

stations of the Pichavaram mangrove ecosystem situated along southeast coast of India. Sediment samples were collected from the rhizosphere of the mangrove plants. The soil samples were serially diluted by the 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 27⁰ 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 Pipet-ting, then 30µl of 10% SDS and 3µl of 20 mg ml⁻¹ proteinase K were added and incubated for 1 hour 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 into 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 incubated at 55°C for 15-20 minutes. The mixture was centrifuged at 23183g for 15 minutes, during which time the contaminants were 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 Terminator 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°C for 4 min] then ramp to 4°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 deionized water and 30 ml of 100% isopropanol was added and mixed by vortex 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 on NCBI server. The alignment of the sequences was done by using CLUSTALW.

Phylogenetic analysis: A phylogenetic analysis of the isolate was performed to determine how the 18S rRNA sequence of the isolate and related strains 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 in 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: i. 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; ii. when percentage similarity is between 95 – 99 %, the unknown isolate would be assigned to the corresponding genus; iii. 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 scale waste was washed with tap water, then distilled water. Thereafter, the wastes were exposed to water vapor and air dried at room temperature¹¹.

Inoculum and cultivation: *Bionectria CBNR BKRR* 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 *Bionectria CBNR BK RR* was prepared by washing 5 days old culture slants with sterilized saline solution (0.9% NaCl) and shaking vigorously for 1 min. The organism was allowed to grow in 100 ml aliquots of mineral salt medium of the following composition (g l⁻¹) : waste, 20; (NH₄)₂SO₄, 2; K₂HPO₄, 1; MgSO₄.7H₂O, 0.5; KCl, 0.5; NaCl, 5; CaCl₂, 0.02; FeSO₄.7H₂O, traces and pH-6 and dispensed in 250 ml Erlenmyer flasks. 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 7463 g for 15 min in a cooling centrifuge. The clear supernatant was used to determine extracellular protein and considered as a crude enzyme to assay chitinase activity¹².

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. 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 an indication to the quantity of released N-acetylglucosamine (NAGA), was measured spectrophotometrically at 575 nm. The amount of NAGA was calculated from the standard curve of NAGA. Chitinase activity (u/min) = the amount of enzyme releasing 1 μmol NAGA per min from colloidal chitin, under the assay conditions¹³. 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 figure-1. The isolates were identified based on the colony morphology, microscopic observation¹⁴ and molecular identification¹⁵.

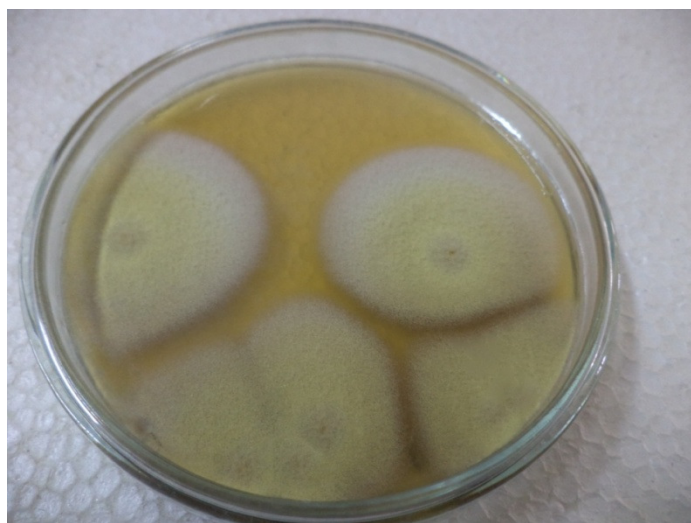


Figure-1
Bionectria CBNR BKRR

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 98% homology with *Bionectria* spp. The sequence so obtained was submitted to the Gene Bank under the accession number KF680540. The entirely new species was named as *CBNR BKRR*. The phylogenetic tree was constructed with the help of Mega5 drawn by neighbor-joining tree using Kimura-2-parameter with 1000 bootstrap replications. The

phylogenetic relation was determined as in figure-2. The homology assay result indicated the isolates were in the phylogenetic branch of *Bionectria* spp.

Chitinase production from various marine wastes: The fungus *Bionectria CBNR BKRR* was grown under shaker conditions at 150 rpm for 5 days in 100 ml liquid medium dispensed in 250 ml Erlenmeyer flasks. The first 5 days represent the logarithmic growth phase of *Bionectria CBNR BKRR* where the enzyme activity is found to be optimum. The highest chitinase activity recorded was 5.8 U/min in the case of Fish Scales wastes and it was consistent with extracellular protein (about 15.5 mg/100 ml) produced by the fungus. The chitinase activity was found to be 4.0 U/min in the case of Shrimp shell waste while its extracellular content was found to be 1.8mg/100 ml. Snail shell waste showed chitinolytic activity of 3.9 U/min and its extracellular protein content was 0.3 mg/100ml. The lowest chitinase activity was observed in the case of crab shell waste (3.7 U/min) and its extracellular content was 0.7 mg/100ml.

Biodegradation studies: The chitinolytic enzyme activity on the different types of substrates was studied and their biodegradation ability was tested; 2g of marine wastes (Crab shell, Snail shell, Shrimp shell, Fish scale) was added to 100 ml of the medium in 250 ml Erlenmeyer flasks. The amount of biomass degraded after 5 days incubation was as follows-Crab shell (0.27g), Snail shell (0.01g), Shrimp shell (0.42g), Fish scale (1.64g).

Discussion: The active component involved in the biodegradation and conversion processes during composting is the resident microbial community, among which fungi play a very important role¹⁶. The biomass ratio of fungi to prokaryotes in compost is about 2:1¹⁷. In addition, fungi use many carbon sources, mainly lignocellulosic polymers and can survive in extreme conditions. They mainly are responsible for compost maturation¹⁸, monitoring fungal diversity is essential to detect fungi hazardous to humans, animals and plants and to optimize compost quality standards¹⁹. Cultivation-independent approaches using rRNA gene sequence analysis have been used to explore the taxonomic diversity of soil microbial communities. Recent technological advances in DNA-based methodologies have allowed rapid and accurate identification of fungal and yeast species from a wide variety of samples.

In fungi, chitinases play important biological and physiological roles, containing autolytic, nutritional, morphogenetic, and parasitic roles. Chitinases in mycoparasitic fungi are most commonly suggested to be involved in mycoparasitism²⁰. *Bionectria* sps are plant pathogens that cause seed rot in oil seed rape. Hence, *Bionectria CBNR BK RR* was chosen as the test organism for the study, in an attempt to explore their distinct chitinolytic ability, with respect to their parasitic manifestations.

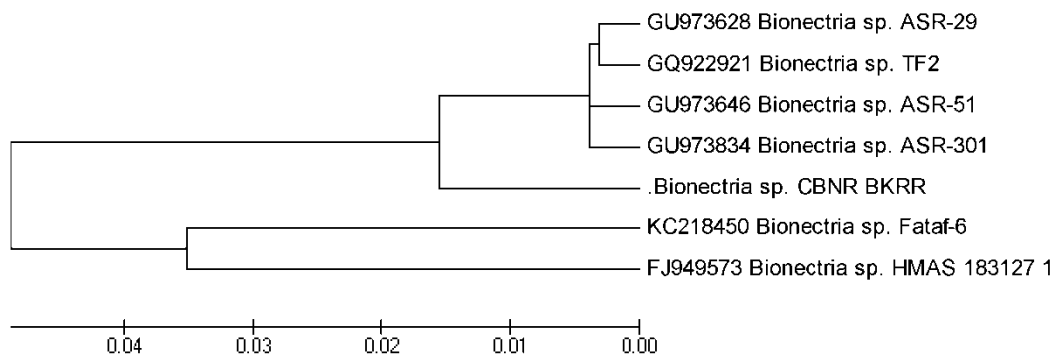


Figure-2
 Phylogenetic tree of *Bionectria* CBNR BKRR showing homology with *Bionectria* spp

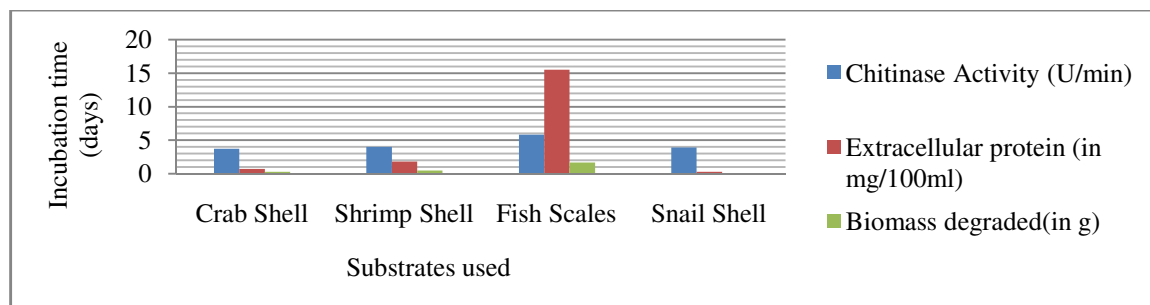


Figure-3
 Chitinase activity, Extracellular protein content and Biomass degraded by *Bionectria* CBNR BKRR in different substrates used

It was indicated that 6 to 7 days of growth were accompanied by the highest chitinase production by *Penicillium chrysogenum*²¹ and *A. alternata*, respectively²². It was reported that 4 days of growth of *Tichoderma harziaum* on shrimp shellfish²³ and *A. terreus* on fish-scales was optimal for chitinase production²⁴. All these findings indicated that highest chitinase production takes place at the logarithmic growth phases of different fungi. It is well known that the highest production of enzymes including hydrolytic enzymes (chitinases) takes place at the accelerated growth phase of microorganisms. These findings are in accordance with the observance of high chitinase activity at the end of 5 days incubation from *Bionectria* CBNR KRRR using different substrate sources.

It was reported that the maximum chitinase activity is 4.309 U/min in case of chitinase production from fish scales wastes from *A. terreus*²⁴. Similar results were also obtained in the case of chitinase production (3.86 U/min) from shrimp shell waste using *A. alternata*²⁵. In accordance with these findings *Bionectria* CBNR BKRR showed high chitinase activity with comparably high concentrations of extracellular protein in case of Fish scales and shrimp wastes while marginal chitinolytic activity was recorded in case of Snail Shell wastes and Crab Shell wastes.

Consequently, higher chitinase production in *Bionectria* CBNR BKRR resulted in accelerated degradation of Shrimp shell and

Fish scales wastes underlying their application in bioremediation of sea food wastes. In harmony with these results, it was found that larger sizes of fish scales were more convenient for higher chitinase production by *Aspergillus* spp. than finest scales²⁴. It was also indicated that as the size of fish scales waste increase, chitinase production by *Aspergillus* sp. decreases²⁴. This due to the differences between constituents and architecture of fish scales and shrimp shellfish wastes and also test organisms.

Conclusion

Our results have demonstrated that the plant pathogen *Bionectria* CBNR BKRR can be effectively utilized for extraction of Chitinase. The degradation of sea food wastes is of much concern which can be averted by successfully optimizing the media conditions to increase production of the enzyme which can be subsequently utilized for bioremediation. This process is cost effective due to the abundance of soil microbes.

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