Production, purification and kinetics of chitinase of *Stenotrophomonas maltophilia* isolated from rhizospheric soil

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Chitinases, through decomposition of chitin, have wide applications, and hence are in demand. Researchers over the period, are looking for potential microbial sources and for optimal production of chitinases. Here, we report isolation of three chitinolytic bacterial species belonging to three genera from different agricultural soil sample collected from Shahada, Maharashtra, India, on minimal agar plates containing colloidal chitin as source of chitin. *Stenotrophomonas* was found to be the most dominant species, followed by *Pseudomonas* and *Alcaligenes*. *Stenotrophomonas maltophilia* identified using 16s rRNA sequencing, Biolog and GC-FAME analysis showed optimum (1.5 U/mL) chitinase activity on chitin agar plates and in submerged culture broth with pH 6-7, incubation of 2 days at 37°C. Presence of CaCl₂ stimulated the enzyme production but EDTA was suppressive. The enzyme upon purification by using sephadex G-100 gel filtration showed improved chitinolytic activity, enzyme kinetics and 2.4 fold increase in purification yield. The molecular weight of purified chitinase as determined by SDS-PAGE was 50-55 kDa.

**Keywords:** Chitinase, Enzyme kinetics, Rhizosphere, SDS-PAGE

Chitinases, through their vital role in decomposition of chitin, have wide range of applications including preparation of protoplasts of fungi and production of oligosaccharides. Chitooligomers produced by hydrolysis of chitin have been used in medical, agricultural and antibacterial, antifungal biological control agent, antihypertensive and, hypocholesterolemic activity, in food industry as quality enhancer and as fibre and textile chemicals.

Though there are many approaches available for improving the production, fold purification and activity of chitinase, reports on kinetic aspects of enzyme activity are scarce. The present work focuses on isolation of chitinase producing organism from rhizospheric soils, their identification and production, purification and characterization and kinetics of chitinase produced by potential isolate.

**Materials and Methods**

**Isolation and screening of chitinase producing bacteria**

Soil samples were collected from rhizosphere of seven different crop fields viz. papaya, cotton, chick pea, chili, banana, wheat and garden soil and isolation of bacteria was done by plating method. Screening for chitinase production was carried on minimal medium containing colloidal chitin 0.5%, Na₂HPO₄ 0.2%, KH₂PO₄ 0.1%, NaCl 0.05%, NH₄Cl 0.1%, MgSO₄·7H₂O 0.05%, CaCl₂·7 H₂O 0.05%, yeast extract 0.05%. Colloidal chitin was prepared according to Roberts & Selitrennikoff. Plates were incubated for 4-6 days at 37°C, and observed for clear zone of chitin hydrolysis around the colony. The colony forming clear zone was selected as the chitinase producer. Potential chitinases producers were selected on the basis of diameter of zone of chitin hydrolysis.

**Estimation of chitinase activity**

Isolate RZS10 showed larger zone of chitin hydrolysis and it was selected for chitinase production in chitin minimal broth for 4-6 days at 37°C at 120 rpm, after incubation broth was centrifuged at 8000×g for 5 min and the supernatant was used for enzyme activities. Chitinase activity was determined by incubating 1 mL of culture supernatant with 1 mL of 1% colloidal chitin in 0.05M phosphate buffer, at pH 7.0 and 37°C for 1 h. After centrifugation of reaction mixture, the amount of N-acetyl-d-glucosamine released in the supernatant was determined by method of Miller using N-acetyl-d-glucosamine (GlcNAc) as a standard. One unit of chitinase activity was defined as the amount of enzyme that liberated 1 µmol of GlcNAc per h under the conditions of the study. Protein estimation was done as per Lowery et al.

**Polyphasic identification of selected isolate**

The selected isolates labeled as RZS10 was subjected to various biochemical tests as per Bergey’s
manual of systematic bacteriology. The pre-sterilized biochemical kits (Kit No. KB 002 & KB 009, Hi Media, Mumbai, India) were used for biochemical tests.

16s rRNA gene sequencing
Genomic DNA was isolated from pure cultures using HiPurA™ Plant Genomic DNA 15. Miniprep purification spin kit (Hi-Media, India). Amplification of 16S rRNA gene sequencing was performed using the primers fD1 (5′-AGAGTTTG ATCCTGGCTCAG-3′) and rP2 (3′-ACGGCTACCTTGTTACGACTT-5′). Amplification was carried out on thermal cycler (Bio-RAD, Hercules, CA) and the amplified product was purified from agarose gel using the Strata Prep® DNA Gel Extraction Kit (Stratagene, Agilent Technologies, USA) and was employed for nucleotide sequencing using Big dye terminator cycle sequencing kit (Applied Biosystems, CA), and ABI Prism 310 Genetic Analyzer (Applied Biosystems, CA). The sequences were analyzed with gapped BLAST (http://www.ncbi.nlm.nih.gov) search algorithm 15.

Whole-cell fatty acid methyl ester (FAME) analysis by MIDI®
The whole-cell fatty acids of bacterial isolates were identified with the help of gas chromatography (GC) using the Sherlock Microbial Identification System (MIDI, Inc. Newark, DE, USA). Qualitative and quantitative differences in fatty acid profiles were used to compute the distance for each strain relative to other strains in the Sherlock bacterial fatty acid ITSA1 aerobe reference library 16-18.

Phenotypic finger printing by BIOLOG system
Carbon-source utilization pattern for 95 carbon sources of strain was analyzed using a BIOLOG system (Microstation, Microbial Identification System, 1998, BIOLOG Inc, CA, USA). About 100 µL of the bacterial suspension (grown in triplicase soya agar) per well of BIOLOG GN III microplate was grown at 30±1°C for 24 h, colour development was read at 595 nm filter in the Microstation reader between 4-6 h and 16-24 h of incubation. Results were compared with the MicroLog software version 4.2 database and the identification acknowledged when the similarity index was ≥0.5.

Enzyme kinetic studies
Effect of pH on chitinase activity
The optimum pH for chitinase activity of purified chitinase of S. maltophilia was determined by incubating the reaction mixture at 37°C for 30 min at various (3-10) pH values. Following buffers were used 0.05M phosphate buffer, (pH 7.0), phosphate buffer (10 mM, pH 7.5) and phosphate buffer (10 mM, pH 7.5) using colloidal chitin as a substrate. Stability of enzyme was checked by pre-incubating the reaction mixture without substrate for 10 min at 37°C, the reaction mixture was immediately cooled down to zero and chitinase activity was measured as described earlier 13-14.

Effect of temperature on chitinase activity
The optimum temperature for chitinase activity of purified chitinase of S. maltophilia was determined by incubating the reaction mixture for 30 min at 37°C at different temperatures (4, 15, 30, 37, 45, 55 and 70°C) at pH 7.5. Thermostability of enzyme was checked by pre-incubating the reaction mixture without substrate for different time intervals (5, 15, 30, 45 and 60 min). After termination of incubation period, the reaction mixture was cooled and chitin was added, enzyme assay was conducted and chitinase activity was measured as described earlier 13-14.

Effect of activator on chitinase activity
The effect of activator and its optimum concentration on activity of purified chitinase of Stenotrophomonas sp. RZS10 was determined by incubating the reaction mixture for 30 min at 37°C with different (100-100 ppm) levels of CaCl2 at pH 7.5. Enzyme stability was checked by pre-incubating the reaction mixture without substrate for different time intervals (5, 15, 30, 45 and 60 min). After termination of incubation period, the reaction mixture was cooled, chitin was added and enzyme assay was conducted and chitinase activity was measured as described earlier.

Effect of inhibitor on chitinase activity
The effect of inhibitor and its optimum concentration on activity of purified chitinase of Stenotrophomonas sp. RZS10 was determined by incubating the reaction mixture for 30 min at 37°C with different (100-100 ppm) levels of EDTA at pH 7.5. Enzyme stability was checked by pre-incubating the reaction mixture without substrate for different time intervals (5, 15, 30, 45 and 60 min). After termination of incubation period, the reaction mixture was cooled, chitin was added and enzyme assay was conducted and chitinase activity was measured as described earlier.

Purification of enzyme
For isolation of substrate specific chitinases, dialyzed protein was charged by affinity binding to
chitin. Cell free culture broth (1L) was precipitated with 10-80% of ammonium sulfate. The pellet obtained after precipitation was washed extensively and incubated for 6 h at 35°C under gentle shaking (90 rpm) to digest colloidal chitin with the absorbed chitinase. The resulting clear supernatant was re-precipitated with 80% ammonium sulfate, centrifuged and dialyzed. The resultant solution was subjected to column chromatography on sephadex G 100 column as per the method of Park et al.19. Fractions adsorbed were eluted and enzyme activity and protein content were determined as described earlier13,14.

Molecular weight determination by SDS-PAGE
Fraction showing chitinases activity was used to determine molecular weight. The molecular mass of the purified chitinase was determined by SDS-PAGE as shown in Laemmli20. Molecular weight of enzyme was estimated by comparing the mobility of the sample with that of standard molecular weight markers of molecular weight in the range 6.0-181.8 kDa. Loaded sample was located by staining the gel with Coomassie brilliant blue followed by destaining21.5.

Results and Discussion
Isolation and screening of chitinase producing bacteria
Amongst 3 potent isolates obtained out of 54, one isolate, identified as \textit{S. maltophilia} later, produced higher (1.5 U/mL) chitinases activity with protein content of 468 μg/mL. As the isolate showed the highest enzyme activity and more protein content it was selected for further study. Many researchers have demonstrated chitinase production in response to chitin or chitosan induction22-23.

Polyphasic identification selected isolate
The comparison of BLAST search of 16S rRNA gene sequences of strains RZS10 with 16S rRNA gene sequences of NCBI GenBank database and phylogenetic analysis revealed highest identity and evolutionary relatedness of strain with \textit{Stenotrophomonas maltophilia} (Fig. 1). The type of fatty acids also matched [similarity index: 0.973] with those present in \textit{S. maltophilia}. The utilization pattern of various biochemicals by the isolate showed maximum similarity index of 0.52 with \textit{S. maltophilia} in the BIOLOG database. Thus, it was identified as \textit{Stenotrophomonas maltophilia}. The gene sequence of isolate was submitted to NCBI GenBank with accession number KR919607 under the name \textit{Stenotrophomonas} sp. RZS10.

Enzyme kinetics studies
Effect of pH on chitinase activity
The kinetic study of chitinases under study revealed that the enzyme was active and stable in the pH range between 6.0 and 7.0, the optimum being 6.5. Enzyme activity ceased below pH 5.0 and above pH 10.0 (Fig. 2). Many chitinases, including the enzyme under study, showed pH optima in acidic range. Decline in enzyme activity can be attributed to the fact that pH of medium affects the ionization properties that not only affect the stability but also the enzyme activity.

Effect of temperature on chitinase activity
Chitinase activity was optimum at 37°C, it decreased at 45°C and inactivated above 50°C and
denatured at 70°C (Fig. 2). Since enzymes are proteins, they are denatured at higher temperatures and lower temperature does not provide sufficient activation energy for catalytic activity of enzyme. Kim et al.21 have reported highest chitinase activity at 30°C from Aeromonas sp. Woo & Park24 have reported maximum chitinase activity at 45°C and stability between 35 and 55°C23-28.

Effect of activator and inhibitor on chitinase activity
Addition of CaCl₂ resulted in an increase in enzyme activity. The enzyme activity was increasing with an increase in the concentration of the activator. Maximum enzyme activity was reported at 1000 ppm of CaCl₂ (Fig. 3). Addition of inhibitor (EDTA) affected the enzyme activity. Higher concentration (900 ppm) of EDTA was threshold level that completely affected the enzyme activity. Many enzymes require metal ions for their optimum activity.

Purification and molecular weight determination of enzyme
Ammonium sulphate precipitate and dialysed extract on sephadex column yielded two fractions. One fraction showed chitinase activity while another fraction did not. This purification method resulted in 2.4 fold purification of enzyme. On SDS- PAGE gel, single band of protein appeared following staining with Coomassie brilliant blue. The molecular mass of the purified chitinase as determined by SDS-PAGE was between 50-55 kDa (Fig. 4). Many reports have claimed the molecular mass of chitinase in the range between 20 to 90 kDa29-35. Kim et al.21 purified chitinase on Sephadex G-100 gel filtration chromatography and reported 20 kDa molecular weight of the enzyme. Ueda et al.27 purified chitinase of Ralstonia sp. on Sephacryl S 200 and reported its molecular weight of 45 kDa. A 40 kDa extracellular chitinase have been purified by ion exchange chromatography and gel permeation chromatography. Chitinases having molecular weight of 50, 59, 67 and 89 kDa from Enterobacter agglomerans have been purified by Yong et al.30.

Conclusion
Stenotrophomonas sp inhabiting the soil is known to produce chitinase that help the organism to lyse the cell wall of fungi, usually phytopathogenic fungi. Isolate with higher chitinase activity and good stability over the range of varying pH, temperature, activator and in presence of inhibitors will have great biotechnological potential in various sectors including agriculture, medical, food and textile.

References


