Production and Some Properties of Chitinase from *Pseudomonas putida* Isolated from Fish Waste Dumping Soil

aN.Saranya, bTha.Thayumanavan  
^a^ Department of Biotechnology, Karpagam University, Coimbatore, India  
^b^ School of Biotechnology, Dr. G.R. Damodaran College of Science, Coimbatore, India

Author for Correspondence:  
N. Saranya,  
Research Scholar, Department of Biotechnology, Karpagam University, Coimbatore – 641 021, Tamil Nadu, India

**Abstract**

Chitin is an insoluble natural polymer, commonly present among crustacean. Fish scales are one of the most common sources of chitin. Chitinase is an enzyme produced by a variety of microorganisms, which can digest the high molecular weight chitin and convert them into low molecular weight and eco-friendly products. Of 60 soil samples collected from various locations of fish scale waste dumping locations, 28 of them were positive for *Pseudomonas* species. Of them, 15 strains have been confirmed as *P. putida*, by 16S rRNA PCR. All the 15 isolates were confirmed as chitinase producers using spot bio assay test on chitin containing media. The maximum chitinase activity was observed at 37 °C and pH 5.

**KEYWORDS:** *Pseudomonas putida*, chitin, chitin degradation, fish scale waste, chitinase.

**Introduction**

About 130 million tons of fish waste is generated each year in the world (Prameela *et al*., 2010). The crustacean waste is the most important chitin source for commercial use due to its high chitin content and ready availability. Chitin holds great economic value due to their versatile biological activities and chemical applications, mainly in medical and pharmaceutical areas. The marine biomass of chitin alone contains approximately 106 – 107 tons. Chitin is closely associated with protein, minerals, lipids and pigments (Park and Kim 2010).

Chitin is a versatile environmentally friendly modern material. It is a naturally occurring high molecular weight linear homopolysaccharide composed of N-acetyl-D glucosamine residues in α (1-4) linkage (Kanafari *et al*., 2007). Chitin, an insoluble linear polymer of N-acetyl glucosamine, is a common constituent of fungal cell walls and of the exoskeletons of arthropods. It is a natural biopolymer with a chemical structure similar to that of cellulose and is a major component of the exoskeleton of invertebrates.

Therefore, crustacean and fish scale waste is ideal as raw material for chitin production. Fish scale set a variety of nutrition, health care, substance-in-one. Zaku *et al*. (2011) successfully isolated chitin from the common crab fish (*Cyprinus carpio* L.). Chitin is extracted from fish scales of Tilapia (*Tilapia nilotica*) to produces chitison from
it (Uawonggul et al., 2002) and also from the scales of *Labeo rohita* (Rui of crap family) by Tanvar et al. (2013).

All organisms that contain chitin also contain Chitinases (EC 3.2.1.14), which are presumably required for morphogenesis of cell walls and exoskeletons. Since plants do not contain chitin in their cell walls, it has been postulated that they produce chitinase to chitin. But they have been found to contain chitinase, often after enzyme synthesis has been induced by microbial infections or other injuries (Walden et al., 1991). Chitinase is often produced in higher plants as a general defense response after wounding or pathogenic attack by Witmer et al. (2003). Chitinase enzymes have their prospective applications in agro-chemical industry, and the microbes capable of producing chitinase are being used effectively against number of soil-borne fungal pathogens and nematodes.

Three potential bacterial strains *Serratia* sp. CN-01, *Serratia* sp. CN-07, and *Pseudomonas* sp. CN-05 were isolated from local environment to develop indigenous resources for the production of chitinase or their use as bio-control agents against plant pathogens (Awais et al., 2011).

Chitinase is best studied enzymes which hydrolyze chitin have broad spectrum of distribution in nature including bacteria, fungi, plants, insects, and protozoa, human, animal and yeast. The roles of Chitinases in these organisms are diverse. In bacteria, chitinases are usually involved in mineralization of chitin nutrition and parasitism. Fungi chitinase however have physiological role in cell division, differentiation and nutritional role (Gohel et al., 2006).

Isolation of chitinolytic bacteria from chitin-rich soil sources therefore would be a powerful approach for selecting bacteria with high chitinolytic activity and to explore the vast diversity of these bacteria from the soil (Subha et al., 2010). *Pseudomonas putida* is a ubiquitous, aerobic, Gram negative bacterium that shows great metabolic versatility. It is adapted to thrive in soils, aquatic systems and the rhizosphere (Dos Santos et al., 2004). They are easy to isolate and grow in the laboratory and they are not fastidious in their requirement of nutrition (Meera, 2009).

*P. putida* strain has been extensively characterized. It has been certified as a biosafety strain, is widely used as an experimental model and is used in biotechnological applications related to agriculture, biocatalysts, and bioremediation and bioplastic production. It is best considered to be the best organism for the isolation of chitin degradation in our experiment. Many chitin-degrading soil bacteria have the ability to inhibit fungal growth (Meera 2009). Nandhakumar et al. (2007) epitomized that the chitinolytic activity of *P. fluorescence*, the chitinase producing ability of the strain with and without the chitin source. The addition of 1% chitin in the culture medium augmented the population of the chitinolytic bacterium. Antifungal activity of chitinases produced by some fluorescent pseudomonads against *Colletotrichum falcatus* went causing red rot disease in sugarcane by Vishvanathan and Swamyappan (2001). *P. putida* has biocontrol agent and effective antagonist of damping off disease such as *Phythium* and *Fusarium* (Validov et al., 2008).

Our objective of the study is to degrade the fish scales wastes in the environment by the isolated organism from a chitin rich soil, by producing chitinase enzymes by the organism and to characterize the enzymes.
Materials and Methods

Collection of samples

About 60 fish scale squander market soil was collected from Palakkad (Kerala state) and Coimbatore and Nagercoil (Tamil Nadu state) for the isolation of *P. putida*.

Sample collection and processing

The samples were collected in a sterile zip-lock cover by using a sterile spatula and transferred into laboratory for the further analysis. About 1g of sample was weighed and serially diluted up to $10^{-9}$ dilution. One ml of the respective dilutions were plated onto the specific media, the King’s B media and incubated at 37°C overnight. The selected colonies were picked and streaked onto a King’s B agar plate to get pure individual colonies. *King’s B medium* which contains 20g of peptone 1.5 g of Magnesium sulphate, 1.5g of dipotassium hydrogen phosphate, 10ml of Glycerol and 1.5g of agar for 1000ml of water with pH 7 (King et al., 1954).

Preliminary identification of bacterial isolates

Gram’s staining, motility determination test, indole test, urease, MRVP test, TSI and tests for catalase, glucose, lactose, maltose and sucrose were done for the preliminary identification of the strain (Paleroni, 1984). Then the strain was subjected to molecular characterization for identification of the specific gene.

Molecular characterization

**Extraction of DNA** (by CTAB/ NaCl solution (10% CTAB in 0.7 M NaCl))

Molecular characterization of the strain was done by isolating genomic DNA (Murray and Thompson, 1980). In 1974, Sanger, designed a procedure for sequencing the DNA similar to the natural process of DNA replication. DNA sequencing enables us to perform a thorough analysis of DNA because it provides us with the most basic information of the sequence of all nucleotides. Identification of strain was done by 16S rRNA sequence analysis. PCR amplification of 16S rRNA gene of *Pseudomonas* sp., was performed to determine the presence of *Pseudomonas* sp. in the template DNA by using the universal forward and reverse primers. Forward: UNIF- AGA GTT TGA TCA TGG CTC AG, Reverse: UNIR-TAC GGC TAC CTT GTT ACG A CTT

PCR

The polymerase chain reaction (PCR) is an enzyme catalyzed biochemical reaction in which stranded DNA (Mullis, 1990). The agarose gel electrophoresis was done with the DNA molecular weight marker to analyze the product.

Degradation of chitin by *P. putida*

The colloidal chitin was prepared by taking of about 5 g of the commercial chitin was slowly added to 100ml of 0.25N HCl with vigorous stirring and kept overnight at 4°C (Roberts and Selitrennikoff, 1988). The ability of the bacterial strain to degrade the chitin was tested by using spot bioassay method on KMB containing 1.0% chitin (v/v) (Viswanathan and Samiyappan, 2001). The plates where then observed for the utilization of chitin.
Production of chitinase

The production of the chitinase in 100 ml King’s B broth was initiated by a loop of the *Pseudomonas* sp., from both the soil sample culture in a 250 ml of conical flask. One percent of the colloidal chitin prepared from the commercial chitin was added to the production media. Then it was incubated in a mechanical shaker under the constant shaking at 150 rpm for about 10 days at room temperature (28 ±2ºC). The maximum chitinase activity was determined between 7 – 8 days. The bacterial cultures were centrifuged at 6,000 rpm for 10 min. The cell free supernatant was collected by passing the above supernatant through the bacterial filtrate. Then it was used for the further analysis.

Determination of protein

Protein estimation was done according to Lowry *et al.* (1951). The blue coloration developed was read at 660 nm and standard graph was drawn. The molecular weight of the compound was determined by SDS-PAGE, the method of Lammeli (1970) in a slab electrophoresis system. Poly peptides separated by SDS-PAGE was fixed with methanol – glacial acetic acid and stained with Coomassie Brilliant Blue R 250. The protein bands were compared with BSA (66 kD) to determine the molecular weight of the marker.

Partial purification

The supernatant was brought to 30% ammonium sulphate saturation by gradual addition in small portions of calculated amount of ammonium sulphate with constant stirring. Then it was dialyzed overnight. Partial Purification was done by Ion Exchange Chromatography with DEAE cellulose. The active fractions were pooled and concentrated using poly ethylene glycon. It was dialyzed against Tris HCl 0.25M buffer.

Characterization of purified chitinase

The purified chitinase was characterized by determining its optimum pH and optimum temperature. Chitinase activity was assayed at different pH values (pH2.0 to 10.0) using different buffers 50 mM such as, Sodium Acetate buffer (pH, 2.6-7.0), Sodium phosphate buffer (pH, 7.5-8.0), Tris-HCl buffer (pH, 7.0-8.5) and Glycine - NaOH buffer (pH, 8.6-10.0). To determine optimum pH, chitinase preparations in buffer at different pH ranging from 4.0-9.0 were kept at room temperature for 1 hour. It was read at 280 nm.

Chitinase activity was assayed at different temperatures ranging from 0ºC - 60ºC at pH 7.5 in Tris-HCl buffer (25 mM). The optimum temperature was determined by incubating them for 1 h. Then the optical density was measured by determining the residual enzyme activity under standard assay conditions.

RESULTS

**Incidence of Pseudomonas sp.**

Incidence of bacterial genera in collected sample was studied with main focus on *Pseudomonas* species. A total of 30 soil samples, 20 marine fish scale soil samples and 10 fresh water fish scale soil samples were collected from various fish markets and processed. In the present investigation, the bacterial genera were inoculated onto their respective selective medium and they showed good growth. Based on the presumptive
identification the bacterial strains were isolated. Among the samples collected marine fish scale showed high percentage of incidence followed by soil sample (Fig.1).

**Isolation and characterization of Pseudomonas species**

Bacterial strains were isolated from the soil and fish scale samples collected from various fish markets. The sample was serially diluted up to $10^{-9}$ dilution and plated on King’s B medium.

**Biochemical identification**

Under morphological analysis and biochemical test the bacterial genera was confirmed to be *Pseudomonas* sp., and for molecular characterizations leads to the conformation of species *P. putida*. The results were tabled in the table (1) shown below.

**Molecular identification**

PCR product was separated using agarose gel electrophoresis and the amplicon size was determined as 1.4 kb of about 1330 bp (Rathanakumari *et al.*, 2012) and this was similar to that of the expected size. Out of 30 isolates 28 shows the expected amplicon size. This molecular identification gives positive results for *Pseudomonas putida*.

**Spot bio assay test**

All the 28 strains show positive results by degrading the chitin containing medium. The strains were able to degrade the chitin by the visible zone formed around the paper disc. The strains to produce a clearance zone as stated by Viswanathan and Swamyappan (2001) were taken for further processing of chitinase production with fish scales as a carbon sources.

**Partial purification**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was done before and after the partial purification. The clear band of 60 kDa for the partially purified enzyme was shown in the fig (2).

**Effect of pH and temperature**

The partially purified enzymes show the wide range of pH from 4.0 to 7.0. But the maximum activity was at pH5. The effect of temperature was also active from 30 °C to 40 °C. The maximum activity was found to be at 37°C for the partially purified sample. It is clearly shown in the fig (3) in a graphical format.

**DISCUSSION**

Isolation of the *P. putida* from the chitin rich soil is able to degrade the chitin source. Subha *et al.* (2010) isolated the chitinolytic bacteria from the chitin rich soil samples. Soil samples are collected from the chitin rich soil which has a history of about not less than 3 years. The chitin contaminated soil samples collected from fish waste dumping sites and in fish markets of South India. The isolated bacterial would be capable of degrading the chin present in the fish scales and produce chitinase enzymes and it is useful for the plants. Moreover selection of the *P. putida* is used for the plants as it is a rhizobacterial and plant growth promoting bacteria. When the *Pseudomonas* is capable of
degrading the fish scales it is used for the biodegradation. Biochemical and molecular characterization was done to the isolated species. The 16S rRNA PCR was done to confirm the presence of *P. putida*. Only 15 isolates gives positive results, as expected amplicon size of total 30 isolated soil samples with a conformed biochemical tests. The amplicon size was similar to study of Rathanakumari *et al.* (2012) with a 1330 bp by using the primers. Isolation chin from the fish scales is not mandatory because of the spot bioassay test by Viswanathan and swamyappan (2001). By using the test we can able to judge the strains for the degradation of chitin with the zone of clearance. Surprisingly all the isolated species shows the positive results to he test because we isolated from the chitin rich soil.

A total of 30 soil samples, 20 marine fish scale soil samples and 10 fresh water fish scale soil samples were collected from various locations. Of them, 65, 50 and 40 % of marine fish scale, soil and fresh water fish scale respectively were positive for *Pseudomonas* sp. So the marine scales were rich in nutrients and the populations were high and we used it for the further processing of our work. The addition of carbon sources other than chitin will support microbial growth. However, it will reduce chitinase production (Felse *et al.*, 2000). Chitinase is produced as an inducible enzyme in the presence of chitin or its degradation products. In most cases, chitin concentration in the range of 1.0-1.5 % (w/v) was found to be most suitable for chitinase production. Suitable chitin concentration is important to ensure that the microorganisms had sufficient amount of carbon source for usage. Nitrogen is the next abundant element in the cell after carbon (Madigan *et al.*, 2000). Chitinase was purified and its molecular weight is of about 60 kDa and it is active at pH 5 at an optimum temperature of 37 °C. Chitinase was isolated from the culture filtrate of *Streptomyces* sp. M-20 was purified and its molecular mass chitinase was 20 kDa by Kim *et al.* (2002). The purified chitinase showed antifungal activity against *Botrytis cinerea*, and lysozyme activity against the cell wall of *Botrytis cinerea*. Chitinase was optimally active at pH of 5.0 and at 30 °C (Kim *et al.*, 2002). Chitinase from *Enterobacter* sp. NRG4 was a 60 kDa extracellular chitinase was purified to homogeneity and characterized. The enzyme was stable from pH 4 to 8, and up to 40 °C. The optimal temperature and pH for activity were 45 °C and pH 5.5, respectively. (Dhahiya *et al.*, 2005).

**CONCLUSION**

Chemical fungicides and pesticides are comprehensively used in current farming practices to protect crops against diseases. However, recently their utilization has been concerned since chemical fungicides and pesticides are highly toxic. They can cause environmental contamination and the presence of fungicide/pesticides residues in food products and induce pathogen resistance. So, biological method is used for the plant against the plant pathogens and lytic enzymes like chitinase producing organism play an important for protection of the plants from disease. The fish scales wastes were the ideal raw material for chitin production. The isolated strains of *P. putida* are possible to degrade the chitin present in fish scale squander and it is an eco friendly organism. By the organism we are able to degrade fish scales and by the same time lytic enzymes were also produced to protect the plants. These results indicated that these bacterial isolates can be used as chitinase producing bio-agents in industry. After few centuries of discovery of chitin, it is widely accepted that this biopolymer is an important biomaterial in many
respects. Numerous studies on chitin have focused on its biomedical applications. However, there is still room for further research. Chitinase genes isolation were also promising role in protection of plant by them.

REFERENCE


Table 1. List of biochemical tests for the identification of isolates

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Biochemical test</th>
<th>Observation</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Indole test</td>
<td>No red colour ring formation</td>
<td>Negative</td>
</tr>
<tr>
<td>2.</td>
<td>Methyl red test</td>
<td>No formation of red colour</td>
<td>Negative</td>
</tr>
<tr>
<td>3.</td>
<td>Voges proskauer test</td>
<td>No pink colour</td>
<td>Negative</td>
</tr>
<tr>
<td>4.</td>
<td>Citrate utilization test</td>
<td>Change of colour from green to Prussian blue</td>
<td>Positive</td>
</tr>
<tr>
<td>5.</td>
<td>Nitrate test</td>
<td>Formation of red colour</td>
<td>Positive</td>
</tr>
<tr>
<td>6.</td>
<td>Catalase test</td>
<td>Production of the gas bubbles</td>
<td>Positive</td>
</tr>
<tr>
<td>7.</td>
<td>Gelatin liquefaction test</td>
<td>Liquefaction of the gelatin</td>
<td>Positive</td>
</tr>
<tr>
<td>8.</td>
<td>Casein hydrolysis</td>
<td>Formation of clearance zone</td>
<td>Positive</td>
</tr>
</tbody>
</table>

Fig 1. Incidence of bacterial species Marine fish samples shows the high growth when it was compared with the soil samples and fresh water.

Fig 2. SDS PAGE before and after purification, this fig shows the clear band of 60 kDa for the partially purified enzyme.
Fig 3. Effect pH and temperature partially purified enzymes shows the wide range of pH from 4.0 to 7.0. But the maximum activity was at pH 5. The effect of temperature was also active from 30 °C to 40 °C. The maximum activity was found to be at 37 °C for the partially purified sample.