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Isolation and Identification of Chitinase Producing Native Fungi From Saltpan of Puthalam, Kanyakumari District, Tamil Nadu, India

George Aldous Jenin[1], Mariavincent Michael Babu[2], Manavalan Murugan[3], Thanigaimalai Murugan[3*]

[1]Department of Biochemistry, Lekshmipuram College of Arts and Science, Neyyoor- 629 252, Tamil Nadu, India.
[2]Centre for Marine Science and Technology, Manonmaniam Sundaranar University, Rajakkamangalam – 629 502, Tamil Nadu, India.
[3]Centre for Biological Science, Noorul Islam Centre for Higher Education, Kumaracoil- 629 180, Tamil Nadu, India.

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ABSTRACT

The objective of the present study is isolation and identification of native chitinolytic fungal strains from infected *Artemia* collected for salt pan of Puthalam, Kanyakumari District, Tamil Nadu. A total of 10 fungi (J1 to J10) were isolated from homogenized *Artemia* sample and screened for chitinase activity on chitin agar plates. In this study, 5 fungi (J1, J3, J4, J5, & J8) showed positive result of chitinase activity and 2 best strains (J1 & J5) were selected for further study. Fungi J1 and J5 were identified as *Aspergillus niger* and *Aspergillus fumigatus* respectively. Greater quality of the enzyme production was achieved with the above strains and the molecular weight of the chitinase was determined by SDS-PAGE it was found to be around 23 KDa. Finally, degradation property of enzyme was assayed with chitin shell waste powder and observed that, degradation activity was higher in shrimp shell powder followed by Prawn shell. The fungal strains such as *A. niger* and *A. fumigatus* have been identified as good chitinase producers.

1. INTRODUCTION

Microorganisms produce many secondary metabolites includes enzymes, pigments, antibiotics which could be of importance to mankind in many ways [1]. Microbial enzymes have several advantages over the enzymes resulting from plants or animals by high merit of their great variety of catalytic activities [2]. Bacteria and fungi are the source for several industrial enzymes includes DNase, lipase, alginase, proteases, chitinases and glutaminase [3, 4]. Chitinase is secondary metabolites produced by a number of naturally occurring microbes. In recent years, the demand for chitinase enzymes with new or attractive properties has augmented due to industrial application of chitin, chitosan and chitooligosaccharides [5]. Chitin is a β 1-4 linked homopolymer of N-acetylglucosamine, it is the second most abundant biodegradable polymer, can be found as a part of fungi, plants, crustaceans, insects, arthropods, and algae components [6]. Approximately 75% of the total weight of shellfish, such as shrimp, crabs and krill are considered as waste, and chitin comprises 20 to 58% of the dry weight of the said waste [7]. Chitinase play important role in the treatment of chitin waste. The chitin wastes especially derived from sea-food-processing units cause remarkable environmental problems worldwide and the production chitinolytic enzyme is an important element to the utilization of shellfish wastes [8]. Several microorganisms, including bacteria such as *Serratia marcescens*, *Bacillus lichiniformis*, *Bacillus subtilis*, *Bacillus thuringiensis*, and *Vibrio alginolyticus* [9-11] and many species of fungi such as *Asergillus sp.*, *Myrothecium anisopliae*, *Streptomyces sp.*, *Trichoderma harzianum*, *Trichoderma viride*, and *Verticillium lecanii* [12-17] have chitinase producing ability. The aim of the present study is isolation and identification of native chitinolytic fungal strains.

** Corresponding Author*
Thanigaimalai Murugan, Centre for Biological Science, Noorul Islam Centre for Higher Education, Kumaracoil- 629 180, Tamil Nadu, India
Email: muruganbt@gmail.com

2. MATERIALS AND METHODS

2.1. Collection of sample

An infected *Artemia* (*A.parthenogenetica)* was collected from saltpan of Puthalam, Kanyakumari District, Tamil Nadu, India. The sample was collected in sterile glass container and immediately brought to the laboratory for further work.

2.2. Processing of sample

The collected sample was rinsed with sterile distilled water for several times to remove soil and other debris and then filtered by muselin cloth filters in order to remove excess liquids. After, about 1 gm of the sample was homogenized with 5 ml of sterilized saline water using morter and pestle.

2.3. Isolation of fungal strains

The homogenized *Artemia* sample was used for isolation of fungi. An aliquot of 0.1 ml of homogenized sample was spread evenly over the surface of Potato dextrose agar (PDA) and Sabouraud dextrose agar (SDA) medium using L-rod. Then, the plates were incubated at room temperature for 3 to 5 days. After incubation, the morphologically different fungal colonies were subcultured into PDA agar plates.

2.4. Screening of chitinase activity

The isolated fungal strains were screened for chitinase activity on chitin-agar medium. 1000 ml of the medium consist of $(NH_4)_2$ SO_4 (1g), K_2HPO_4 (1g), KCl (0.5g), NaCl (5g), $MgSO_4$ (0.5g), $FeSO_4$ (0.01g), agar agar (20g) and colloidal chitin (5 g). A portion of isolated fungus inoculated on chitin agar plates, incubated at room temperature for 6-8 days. The chitinase activity was observed by the halo zone of clearances around the fungal colonies, further it may be confirmed by adding Congo red dye solution.

2.5. Characterization and identification of chitinolytic fungi

The chitinase producing fungi were subjected to identification based on cultural and morphological characteristics. Growth colour, texture and pigments were observed macroscopically and structure of the fungi was observed by microscopically using lacto phenol cotton blue staining [18, 19].

2.6. Production of chitinase

Production of chitinase enzyme was carried out in 250ml of conical flask with 50ml of production medium, which consist of the following constituents (g/L) $(NH_4)_2$ SO_4 (1g), K_2HPO_4 (1g), KCl (0.5g), NaCl (5g), $MgSO_4$ (0.5g), $FeSO_4$ (0.01g), and colloidal chitin (5 g). The flasks were inoculated with 1 ml of fungal suspension and then incubated at 30°C under shaking condition (160 rpm) for 7 days.

2.7. Purification of enzyme

The crude enzyme suspension was precipitated with 75% (w/v) of ammonium sulphate. The mixture was kept for overnight at 4°C. The pellet was recovered by centrifugation and dissolved in 50 mM phosphate buffer (pH 7.0). The suspension was dialyzed against distilled water for 24 hours and the solutions obtained after dialysis was loaded on Sephadex G-100 columns for further purification to standardize homogeneity levels. 100 mM phosphate buffer was used as elution buffer and the fractions were collected at flow rate was 0.5 ml/ min.

2.8. Estimation of chitinase activity and protein concentration

Chitinase activity was determined spectrophotometrically by estimating the amount of free reducing groups formed after colloidal chitin hydrolysis. The reaction mixture consist of 1% colloidal chitin (suspended in 0.02 M phosphate buffer pH 7) 0.5 ml of enzyme solution. The mixture was incubated at 30^{o}C for 1 hour. The product was measured in 1 ml of filtrate by dinitrosalicylic acid (DNS) method using N-acetylglucosamine as standard [9]. Total protein content of the enzyme was estimated by the method of Lowry *et al.* [20] using bovine serum albumin (BSA) as standard.

2.9. Molecular weight determination

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12% polyacrylamide gel, by the method of Laemmli [21]. The sample was treated with sample buffer and boiled at 100°C for 3 min before loading. A voltage of 100 V was applied and the gel was left running for 45 minutes to achieve sufficient migration. Proteins were visualized by staining the gel with Coomassie blue G-250 and the molecular weight of the enzyme was determined by comparing with the bands of standard marker protein.

3.10. Study of degradation activity

Degradation activity of chitinase was performed with various chitin shell waste *viz*., Shrimp shell, Prawn shell, Crab shell and Squid shell. Potato dextrose agar plate was prepared with 2% chitin waste powers. The medium was autoclaved at 121^{0}C for 20 minutes and poured into sterile Petri dishes. After solidification, well (about 6mm in diameter) was made on the surface of agar plate using well cutter. After, about 50 µl of crude enzyme suspension was poured into the well in an aseptic manner. Then, the plates were incubated at 30^{0}C for about 10-15 days and/ or observed periodically to ensure the zone formation around the well.

3. RESULTS

3.1. Isolation of fungal strains

In this present study, a total of 10 different fungal colonies were recovered from the homogenized sample of infected *Artemia.* All the colonies were individually subcultured on PDA agar plates and screening of chitinase activity was performed.

3.2. Screening of chitinase activity

The presence of clear halo around the colonies indicates the chitinase activity of the fungus. In this investigation, five fungal strains (J1, J3, J4, J5, & J8) showed positive result for chitinase production. Among five, 2 best strains such as J1 & J5 were selected for further study.

3.3. Characterization and identification of chitinolytic fungi

The best two strains were identified by studying cultural and morphological characteristics (Table 1). Based on the

characters studied, the strain J1 and J5 were identified as *Aspergillus niger* and *Aspergillus fumigatus* respectively.

Table 1: Cultural and morphological characters of fungi.

SL. No.	Characteristics	*A. niger*	*A. fumigatus*
1	Colony color	Black	Pale green
2	Colony appearance	Spread spores	Spread spores
3	Colony texture	Smooth	Smooth
4	Surface of the colony	Smooth colony	Smooth colony
5	Conidial head	Present	Present
6	Sporangium	-	-
7	Stipe	-	-
8	Phialides	-	-
9	Conidia shape	-	-
10	Vesicles shape	Ovaid	Ovaid
11	Vesicles arrangement	Biseriate vesicles	uniseriate vesicles
12	Appearance of Metula	Entirely covering medulla	Upper 2/3 covering medulla
13	Medulla shape	Oval shape	Glubose shape
14	Rhizoids	-	-

3.4. Production of chitinase

The chitinase was produced by using best two strains. After 7 days of incubation, the flask containing production medium was centrifuged and the supernatant containing crude enzyme was used for further studies.

3.5. Estimation of chitinase activity and protein concentration

The enzyme activity was estimated calorimetrically and the values calculated using standard values of standard N acetyl glucosamine. Total protein content was calculated based the values of standard bovine serum albumin. The enzyme activity and protein concentration of the chitinase enzyme produced by *A.niger* and *A.fumigatus* mentioned in table 2.

Table 2: Total enzyme activity and protein concentration of chitinase.

SL. No.	Strain Name	Total activity (µg moles/ml/min)	Total protein (mg/ml)
1	*A.niger*	0.612	21
2	*A.fumigatus*	0.575	18

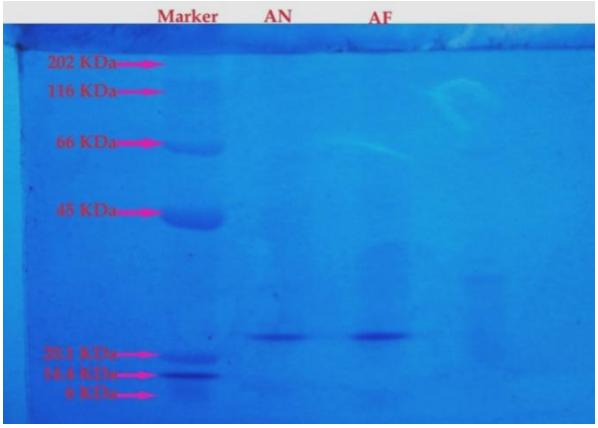

Fig 1: SDS PAGE gel of chitinase enzyme

3.6. Molecular weight determination

The protein band of chitinas enzyme was observed and it was compared with the bands of standard marker proteins. In this study, the molecular weight of the chitinases from *A.niger* and *A.fumigatus* were found to be around 23 KDa (Fig. 1).

3.7. Study of degradation activity

It was observed that, the chitinase produced by *Aspergillus fumigatus* have the ability to degrade chitinase waste. The degradation activity was higher in shrimp shell powder followed by Prawn shell. In crab shell and Squid shells, the enzyme showed moderate degradation (Figure 2).

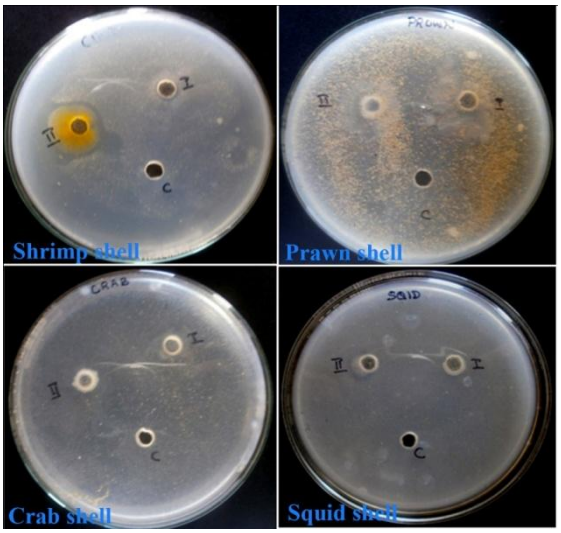

Fig 2: Degradation activity of chitinase

4. DISCUSSION

Chitinases are widely distributed in microorganisms, the filamentous fungi including *Trichoderma, Oenicillium, Penicillium, Lecanicillium, Neurospora, Mucor, Beauveria, Lycoperdon, Aspergillus, Myrothecium, Conidiobolus, Metharhizium, Stachybotrys and Agaricus* [22-24].

In this present study, the two fungal species belonging to the genus *Aspergillus* such as *A.niger* and *A.fumigatus* were found to be good chtinase producers. In those two strains, the first one i.e *A.niger* showed more enzyme than *A. fumigatus*. Sharaf [25] reported that the genus *Aspergillus* is comprised of *A. flavus, A. foetidus, A. niger and A. ungius*. *A. flavus* and *A. alternata* which were developed on the plates containing colloidal, treated and crude chitin, exhibits a strong chitinolytic activity. In this present study, colloidal chitin prepared from chitin shell powder was used as substrate and more quantity of chitinase enzyme was achieved. Purification of chitinase enzyme produced by *Aspergillus* species was done by chromatography. The molecular mass of the enzymes were studied by SDS-PAGE using 10% acrylamide gel and coomassive brilliant blue R250 as staining reagent. These purified chitinases were probably new since the molecular mass was found to be 23 KDa, is different from the previously published values of 33, 43.5, and 45 kDa [26, 27].

Chitin is one of the most abundant polysaccharides on the earth. A lot of chitineous substances are found in the shells of shrimp, crabs, lobsters [28]. However, these substances are

discarded as wastes and its degradation is of great importance as it can supply to both carbon and nitrogen cycles in the biosphere [29]. The ability of bacteria, fungi, and actinomycetes to produce chitinolytic enzymes is common in nature. The great majority of these organisms are able to effectively break down chitin and use it as a source of carbon and energy and also to produce chitinases [30]. In this present study, degradation activity of chitinase enzyme was studied against various chitins containing substances. The degradation activity was higher in shrimp shell power followed by Prown shell. Also, the degradation occurred after 8 days of incubation. This result was supported by the studies of Rattanakit *et al.* [31] and Hoang *et al.* [32].

5. CONCLUSION

The fungal strains such as *Aspergillus niger* and *Aspergillus fumigatus* have been identified as good chitinase producers isolated from infected *Artemia* collected from the saltpan of Puthalam. It was confirmed that, the purified enzyme was able to degrade chitin waste substances. In future interest will be taken to scale up the production of chitinase by using various agro residues in solid state fermentation.

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