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Evaluation of Chitinase producing and antimicrobial properties of *streptomyces* isolated from shrimp shell disposable area.

Subramanian Kumaran* Balaraman Deivasigamani*, Uttara Vairagkar, Sadaippan Balamurugan & Manikkam Sakthivel
 CAS in Marine Biology Faculty of Marine Sciences Annamalai University Parangipettai Tamil Nadu, India

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ABSTRACT

Objective: At present scenario need the potential medical application enzymes and control the major clinical bacterial pathogens produce infections in humans. In this mind isolate and characterization of chitinase and antibacterial compounds from streptomyces. **Methods:** The actinobacterial strains isolated from crab and shrimp shells disposable area (Parangipettai) were screened for chitinolytic activity on colloidal chitin–agar plates. After incubation the clear zone producing strain were selected for chitinase production. Every 2 days interval chitinolytic activity was measured and protein estimation was determined. Antagonistic activity of chitinase producing actinobacterial isolates were tested by adopting agar plug method. Based on the preliminary screening results, chitinase producing strain was used for bioactive substance production through submerged fermentation by adopting shake flask method. Antibacterial activity of crude extracts was tested by disc diffusion method at 100 µg/disc concentration. **Results:** totally 15 actinobacterial strain were isolated. Based on the zone formation on chitin minimal plate CDB20 showed promising zone activity. The CDB20 strain was inoculated in chitin production medium at 10 days. After incubation the chitinase activity showed 1.22U/ml protein estimation 14mg/ml. The preliminary screening showed promising antibacterial activity against clinical bacterial pathogens. Secondary screening results showed maximum *K. pneumonia*(23mm) and minimum *S. aureus* (18mm) was observed. The potent strain identified as genera streptomyces. **Conclusion:** The isolated potent streptomyces have degraded chitin and inhibited the clinical bacterial pathogens. In future in this strain will be used for waste shrimp and crab shells management and recycling and In this strain produce chitinase and antibacterial compounds have more medical application. So this strain has multi functional applications.

1. Introduction

Chitin is one of the most abundant natural polysaccharides in the world. Global production of crustacean shellfish has been estimated to be 1.9 million metric tonnes [1]. During processing of shrimps its head, shell and tail portions are removed which generates 1.44 million metric tonnes of protein and chitin rich substances every year [2]. Thus, attention must be paid to greater utilization of shrimp processing by-products in order to address such concerns. Shrimp waste contains protein and chitin in range 33–40 and 17–20% on dry weight basis, respectively and hence can serve as a cheap substrate for microbial protease and chitinase production [3]. Chitinases, a group

of enzymes capable of degrading chitin directly to low-molecular-weight products, have been shown to be produced by a number of microorganisms. Almost all of the reported chitinase-producing strains will use chitin or colloidal chitin as a carbon source. The production of inexpensive chitinolytic enzymes is an important element in the utilization of shellfish wastes that not only solves environmental problems but also promotes the economic value of the marine products [4].

Streptomyces are soil-dwelling mycelial bacteria that produce a large number of secreted proteins and many secondary metabolites, including important antibiotics. Chitin is a major nutrient source for many streptomyces, and these microorganisms have developed complex extracellular systems for chitin utilization [5].

Among the various industrially important microorganisms, actinobacteria are of prime importance and are primarily recognized as organisms of academic curiosity and also as potential antibiotic producers. Actinobacteria are the

*Corresponding author: Contact information CAS in Marine Biology Faculty of Marine Sciences Annamalai University Parangipettai – 608502 Tamil Nadu India
 Tel : +91-9943215332; +91-9443880023
 E-mail: kumarun23@gmail.com; b.deivasigamani@gmail.com

common inhabitants of soil with an unprecedented ability to produce numerous high value metabolites including the antibiotics of clinical importance [6]. It was found that most of the actinomycete isolates possessed high proteolytic, cellulase and chitinase activity and it was also mentioned about an antibacterial one.

In shrimp and crab culturing industry waste affect the nature ecosystem. In future it's become major problem of pollution. So now urgent we need management and recycling of chitin using bacterial chitinase and its have more biomedical value. In this present investigation to isolate and Characterize of the potent actinobacterial chitin degrader and antimicrobial metabolites

2. Materials and methods

2.1. Screening and Culture Condition

The actinobacterial strains isolated from crab and shrimp shells disposable area (Parangipettai) were screened for chitinolytic activity on colloidal chitin–agar plates (0.2%, w/v colloidal chitin) and aged sea water. Chitinolytic activity was expressed by zones of clearance around individual colonies. One strain (CDB20), which showed the largest zone of clearance, was used for further analysis.

2.1. Chitinase Production

The chitinolytic enzymes production medium was colloidal chitin 5 g, (NH₄)₂SO₄ 7 g, K₂HPO₄ 1 g, NaCl 1 g, MgSO₄ .7H₂O 0.1 g, tryptone 1 g, yeast extract 0.5 g Distilled water 1000 ml, pH 8.0 [7]. Inoculum was prepared by dispersing strain CDB20 spore suspension into 50 ml of production medium in 250 ml Erlenmeyer flask and incubated for 10 days at 28 °C, 100 rpm. Every 2 days interval, chitinolytic activity was determined by chitinase assay by centrifugation at 10,000 × g for 20 min.

2.3. Chitinase assay and protein estimation

The assay mixture contained 1 ml chitin (12 mg/ml 20 mM Tris / Hcl buffer, pH 8.0) and 0.5 ml enzyme solution. After incubation at 55°C for 1 h, it was centrifuged at 3000 g for 10 min. The amount of N-acetyl- D-glucosamine (GlcNAc) released in the supernatant was determined using GlcNAc as the standard. One unit was defined as the amount of enzyme that catalyses the release of 1 mmol GlcNAc in 1 h at 55°C.

Protein content was determined by measuring the absorbance at 280 nm during purification. For all other purposes protein was measured by the method of [8] using bovine serum albumin as the standard.

2.4. Screening for antagonistic activity

Antagonistic activity of chitinase producing actinobacterial isolates were tested by adopting agar plug method. Test bacterial pathogens used in this study include, *S. aureus*, *B. subtilis*, *K. pneumonia*, *E. coli* and *P. aeruginosa*. All the bacterial isolates were obtained from the Department of

Microbiology, RMMC, Annamalignagar, Chidambaram. The absence of an inhibition zone indicated a negative result for the production of diffusible metabolites in to the solid growth medium [9].

2.5. Production of bioactive substances

Based on the preliminary screening results, chitinase producing strain was used for bioactive substance production through submerged fermentation by adopting shake flask method. The isolated actinobacterial strain inoculums was transferred into each 100 ml of ISP2 broth and incubated in rotary shaker with 110 rpm at 28°C for 7 days. After incubation the cell free supernatant was separated by centrifugation at 10000 rpm for 10 minutes [10].

2.6. Extraction of compound

The cell free supernatant which showed maximum zone of inhibition in well diffusion assay was extracted using equal volume of solvents such as ethyl acetate and n-hexane for overnight. Then the solvent portion was collected and concentrated by evaporation. The 5 clinical bacterial were used for antibacterial activity. Antibacterial activity of crude extracts was tested by disc diffusion method at 100 µg/disc concentration [11].

2.7. Characterization of potent isolate

Cultural characteristics of selected actinobacterial isolates were studied by inoculating them in to ISP2 agar medium. After 7 days of incubation, cultural characteristics such as growth rate, consistency, aerial mass color, reverse side pigment, soluble pigment were recorded.

3. Results

A total of 15 chitinolytic actinobacteria were isolated from crab and shrimp shells disposable area, Tamil Nadu. All the isolates were isolated using colloidal chitin minimal medium. Among the 15 chitinolytic actinobacteria only CDB20 strain showed zone of clearance. The CDB20 for used for further characterization. The specific activity of chitinase (Units/ml/minute) was determined by using colloidal chitin prepared from standard chitin. Standard graph was prepared by N-acetyl glucosamine as standards and the concentration of N-acetyl glucosamine obtained as the product after degradation of colloidal chitin from each of the substrates was determined by DNS method. The potent actinobacteria strain was degraded to chitin in the production medium. After centrifugation, culture supernatant was screened for chitinase activity. The results showed 1.22 U/ml (Figure 1). The potent isolate utilize chitin for sole carbon source.

Growth of the isolates was measured in terms of protein level 14mg/ml. Antibacterial activity of isolated chitin degrading actinobacteria was preliminarily screened by agar plug method against clinical bacterial pathogens. The results showed the CDB20 strain moderate inhibition of all

the bacterial pathogens. So we conclude this is considered for drug development in future. So need to further characterization of the crude extract.

Table 1.
Isolated CDB20 Characteristics.

Characters	Isolated strain CDB20
Aerial mycelium	+
Substrate mycelium	+
Spore chain morphology	RF
Colony consistency	Powdery
Aerial mass color	Pink
Reverse side pigment	Brown
Soluble pigment	Brown
ISP1	Good
ISP2	Good
ISP3	Good
ISP4	Good
ISP5	Good
ISP6	Good
ISP7	Moderate
ISP7	Moderate
Glucose	+
Xylose	+
Mannitol	+
Sucrose	–
Rhamnose	+
Inositol	+
Fructose	–
Raffinose	–
Cellulose	+
Arabinose	–

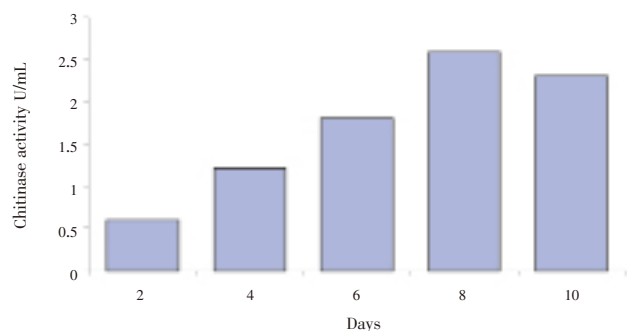


Figure 1. Chitinase assay

The secondary screening of actinobacterial crude extract was carried out. After 5 days incubation growth was observed. The culture supernatant was used for extraction and characterization. The culture supernatant was extracted by ethyl acetate. After solvent evaporation, crude extract dissolved in buffer and its analysis of antibacterial activity. Amongst the test bacterial cultures the compound showed (Figure.2) inhibition against, *S. aureus* (18mm), *B. subtilis* (21mm), *K. pneumonia*(23mm), *E. coli* (22mm) and *P. aeruginosa* (20mm). Under microscopic observation, strain CBD20 showed the presence of substrate and aerial mycelium with rectus flexible (RF) arrangement of spore chains. Cultural characteristics of strain CDB20 was given in

table 1. Based on the result the strains suspected genera for strptomyces.

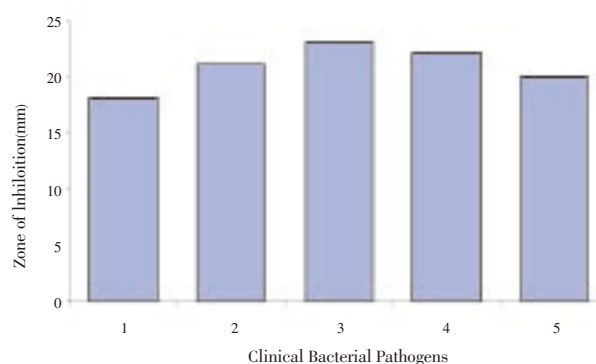


Figure 2. Antibacterial activity of crude extract by disc diffusion method.

1. *E. coli*, 2. *S. aureus*, 3. *B. subtilis*, 4. *K. pneumonia* and 5. *P. aeruginosa*

4. Discussion

The Chitinolytic systems of Streptomyces are usually composed of a diversity of enzymes with different specificities. Hoang [12] *et al.*, (2011) stated the chitinase from microorganisms are extremely important for the degradation and recycling of the carbon and nitrogen trapped in the large amount of insoluble chitin in nature. In this study, one actinobacteria showed clear zone on chitin plates out of 15 strains. So the isolate degrade the chitin though chitinase enzyme. Already Hoang [12] *et al.*, (2011) reported Streptomyces sp. TH-11 was isolated from the sediment of the Tou-Chien River, Taiwan. In this finding we isolated streptomyces from shrimp and crab shells disposable area. In this work we made new attempt checked for antibacterial activity also for chitinase producing actinobacteria. No more records for the chitinase producing actinobacterial compound inhibits the clinical bacteria. The chitinase have strong medical and industrial application. Chitinase is widely used for example in preparation of pharmaceutically, Drug delivery, biopesticides and mosquito control [13]. New founded research says that chitinase has potential as a serum for human [14].

The isolate was inoculated on chitin minimal medium for 10 days. Every 2 days interval chitinase were estimate. Initial 2 days to 8 days its gradually increased after 10th day its decrease.

The protein estimation result 14mg/ml showed protease present in the culture broth. Already reported that the bacterial chitinase and chitosanase production in both substrates medium increased at first 4 d, and the reducing sugar increased at first 5 d, then declined. Chitinases of various molecular weights have been identified in various Streptomyces strains [15]. These including a 20 kDa chitinase from Streptomyces sp. M-20, 28 kDa, 25 kDa, and 45 kDa from Streptomyces sp. NK 1057, 45 kDa and 45 kDa from *S. albovinaceus* S-22, 45 kDa from Streptomyces sp. ANU 6277, and 49 kDa from *S. griseus* HUT 6037 [16].

The actinobacteria showed pigment on cultural plates so we

can try to isolate bioactive metabolites from actinobacteria. The microbial antibiotics discovered, more than 50% of that are produced by the members of the group actinobacteria especially Streptomyces.

The preliminary results showed promising antibacterial activity against clinical bacterial pathogens. The disc diffusion of crude extract result showed promising zone against 5 clinical pathogens. Already reported the streptomyces have inhibited the clinical bacterial pathogens [17]. In our result more effective inhibition rate against bacterial pathogens. The potent isolated strain produces chitinases and antibacterial compounds. Chitinases breakdown chitin into a variety of products that include the deacylated oligomer chitosan (GlcNAc)_n, the disaccharide chitobiose (GlcNAc)₂ and the monomer N-acetylglucosamine. These derivatives are increasingly finding use in diverse fields, such as biomedicine [18]. No more report of the chitin degrading actinobacter produce antibacterial compounds against clinical pathogens.

The isolated streptomyces have more medical application. The strain produces chitinase and antibacterial compounds. The chitinase have more important in medical field such as Drug deliver, Antibacterial agents, Immunoadjuvant, Anti HIV-1 activity etc., another hand is streptomyces compound inhibit clinical bacterial pathogens. So in future it could be helpful for development of natural drug.

Conflict of interest statement

We declare that we have no conflict of interest.

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