ANTIBACTERIAL POTENTIAL OF CHITIN AND CHITIN-BASED DERIVATIVES AGAINST PATHOGENIC AND DRUG-RESISTANT BACTERIAL STRAINS

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ABSTRACT

Crustacean’s shells constitute the traditional and current commercial source of Chitin. Chitin and its derivatives as a potential resource as well as multiple functional substrates have generate attractive interest in various fields such as biomedical, pharmaceutical, food and environmental industries. In the present investigation, chitinous wastes were collected from the fresh water areas of Dehradun and Rishikesh of Uttarakhand State. The bacterium, Bacillus sp. isolated from soil produces chitinase enzyme responsible for degradation of chitin obtained from chitinous wastes. Further the chitinases enzyme was utilized to degrade the chitinous wastes into Chito-oligosaccharides. The chitin active molecule present in the chitinous waste at another stage was deacetylated to chitosan. Further the antibacterial activity of chitinases, chitin, chitosan and chito-oligosaccharides was determined in vitro by well diffusion method. The enzyme purified showed potent activity against the bacterial cultures but no activity was observed against the fungal test cultures. Amongst the test bacterial cultures the chitinase showed maximum inhibition against Micrococcus luteus (diameter of zone of inhibition: 21 mm) followed by multi-drug resistant Staphylococcus aureus (diameter of zone of inhibition: 20 mm) and Salmonella abony (diameter of zone of inhibition: 17 mm). Further, chitin, chitosan and chito-oligosaccharide were subjected to antimicrobial activity against the similar strains and the results were found to be very satisfactory as the chitin and chitin-based derivatives were equally antimicrobial in nature.
KEYWORDS: Chitin, crustaceans, chito-oligosaccharides, chitosan, chitinases, antibacterial activity.

INTRODUCTION
Chitin, a polysaccharide of animal origin, is obtained from waste material of seafood industries. It occurs in the skeletal material of crustaceans such as crabs, lobsters, shrimps, prawns and crayfish. Chitin is also the important component of exoskeleton of Arthropods. Chitin is also forming the important composition of fungus. Chitin hold great economic value due to their versatile biological activities and chemical applications, mainly in medical [1,2] and pharmaceutical areas. [3,4] Chito-oligosaccharides and their N-acetylated analogues are useful for applications in various fields because they have specific biological activities such as antimicrobial activity, antitumor activity, immune-enhancing effects. [5] Some chito-oligosaccharides such as (GlcNAc) and (GlcNAc) have been reported to possess antitumor activity. [6,7] Chitinolytic enzymes have been widely used in various processes including the agricultural, biological and environmental fields. [8] Several chitinolytic enzymes have been identified in various Streptomyces sp., including, Streptomyces plicatus, [9] S. lividans, [10] S. virdificans [11] and S. halstedii. [12] The chitinases were purified and characterized from marine bacterium. [13] The potent chitinolytic activity of marine actinomycetes species and enzymatic production of chito-oligosaccharides was investigated. [14] In the present study, the antibacterial activity of chitin and chitin based derivatives was determined in lieu of determination of pharmacological properties present in the abundant polymer.

MATERIALS AND METHODS
All the materials, reagents and media used for the study were procured from Ranchem, CDH and Hi-Media, India.

Collection of Chitinous Wastes
The chitinous wastes of fresh water crustaceans were collected from the fresh water areas of Dehradun and Rishikesh (U.K), India and were washed properly in order to remove the sand debris present on the surfaces. The chitinous wastes were then after air dried and powdered material obtained was used as chitin.

Demineralization of Chitinous Wastes
The demineralization of chitinous wastes was performed. [15,16] The chitinous wastes were treated with 1.75 N acetic acid at room temperature for about 12-15 hours. The ratio of waste
to solvent were maintained (1:15 w/v). The demineralized material obtained were recovered by filtration and rinsing with de-ionized water and will be dried in forced hot air oven at 650°C.

**Deproteinization and Removal of Lipids**

The new and advanced methodology for deproteinization of proteins from demineralized chitinous wastes was designed by using deproteinization agents. This process can be performed either by using proteolytic enzymes such as proteinase-K dissolved in buffer containing 0.05 M Tris-base (pH, 6.5-9.1) in a ratio 1:20 (w/v) in flasks at various temperatures in incubator-shaker for about 72 h and adding mixture of solvents (phenol: chloroform, 1:1 ratio) again and again to the residue obtained and centrifuging the mixture until the residue gives no test for the presence of protein content. After repeating the procedure for 3-4 times, finally the residue was treated with 2N sodium hydroxide (1:25 w/v) at 700°C for 1 hour. The lipid content gets dissolved in phenol: chloroform mixture and was removed from the chitinous wastes. Grease spot test can be performed in order to determine qualitatively the presence of lipid content if any present in the residual material. [16]

**Preparation of Colloidal Chitin**

The colloidal chitin was prepared by using 1g of standard chitin, fresh water crustaceans chitin separately in 1N HCl for 2 h at room temperature. The colloidal chitin prepared of each of the samples was washed several times with large volume of distilled water to adjust the pH to 7.0.

**Isolation of Microorganisms for Screening of Chitinases Production**

The soil was procured from Doiwala region of Dehradun (U.K), India by performing the serial dilution method and maintained on glycerol yeast medium plates at 37°C.

**Screening and Culture Conditions**

For the screening purpose, strain was inoculated in 100 ml of medium (3% w/v chitin, 0.1% KH2PO4, 0.05% MgSO4.7H2O, 50 mM Sodium Phosphate buffer, pH 6.0) in a 250 ml Erlenmeyer flask at 30°C. Further basic dye cresol red was added in a flask and kept for 18-24 h. The conversion of colour of the red dye into purple (pH, 6.5- 8.8) was taken as an indication for the presence of Bacillus sp. Further biochemical tests such as amylase production/starch hydrolysis assay were performed in order to confirm the strain as Bacillus sp. Gram staining confirmed the strain as gram positive. [16]
Chitinase Production
For the production of chitinase, strain was grown in 100 ml of fresh medium (3% w/v chitin; 0.1% KH2PO4; 0.05% MgSO4.7H2O; 50mM Sodium Phosphate buffer, pH 6.0) in a 250 ml Erlenmeyer flask at 30°C. For reflecting the growth of the culture in this medium OD at 660 nm was taken using blank as medium in which no inoculum was added. The supernatant (enzyme) was collected from 3 day old cultures by centrifuging the mixture at 12,000 g for 20 minutes. The enzyme was concentrated by condensing the solution in order to reduce its volume. [16]

Preparation of Chito-oligosaccharides
For the preparation of chito-oligosaccharides, 1% colloidal chitin prepared from standard chitin, fresh water crab and fresh water prawn were dissolved in 0.05M Phosphate buffer (pH, 5.5). About 10 ml of enzyme was added in 15 ml of 1% of each of the colloidal chitin in a 100 ml flask; further flask was kept at 30°C for 3 h. The reaction was terminated by immersing the tubes in boiling water for 5 minutes. After performing centrifugation at 3000 rpm, the insoluble materials were collected and condensed to obtain the chito-oligosaccharides. [16]

Preparation of Chitosan
The demineralized and deproteinized chitin material was subjected to concentrated sodium hydroxide at 40% w/v. [17,18] The deacetylated forms of chitosan obtained were solubilized in 2 M dilute acetic acid.

Determination of Antimicrobial Activity of Chitin and Chitin-Based Derivatives Produced from Strain
The antimicrobial activity of chitinase produced from Bacillus strain and chitin-based derivatives were screened for its antibacterial activity against some standard bacterial strains viz. E.coli, Lactobacillus plantarum, Salmonella abony, Micrococcus luteus, drug resistant Staphylococcus aureus, drug resistant Acinobacter by well diffusion method. [19] The pure cultures of test microorganisms were procured from National Chemical Laboratory (NCL), Pune, Maharashtra, India. Nutrient agar medium/broth was used for the growth of bacterial cultures while Sabouraud’s dextrose agar/broth was used for the fungal cultures. The wells were punctured in the agar plates with sterile borer and 105 Cfu/ml of the bacterial and fungal cell suspension were introduced in the plates separately. The enzyme supernatant was introduced in the wells in each of the bacterial and fungal plates. The plates were left free for
the thorough diffusion of the enzyme supernatant within the medium plates and were kept for 18-24 h and 72 h at 370 C for bacterial and fungal cultures respectively. The diameter of zone of inhibition observed was recorded.

RESULTS AND DISCUSSION

In the present investigation, the chitin, chitosan and chito-oligosaccharides (Chitin-based derivatives) were extracted and purified from crustacean exoskeleton wastes. The chitinases enzyme was purified from Bacillus strain, isolated from soil. The enzyme was utilized to degrade the chitin (extracted from crustacean waste) in order to prepare chito-oligosaccharides. Further, the extracted chitin was subjected to deacetylation process to produce chitosan. These derivatives were dissolved in 0.1% w/v Sodium hydroxide in order to assess the antibacterial activity against the bacterial strains. These all derivatives along with purified chitinases enzyme were subjected for antibacterial activity against the pathogenic and drug-resistant strains. The results were compared with positive controls, chloramphenicol and erythromycin (1 mg/ml). The results are shown in Table 1 and Figure 1 (a) and 1(b). The present study reveals the potent antibacterial potential of chitin and it’s based derivatives as significant antibacterial agent against the drug-resistant pathogens. The results reveal that, chitosan and chito-oligosaccharides had significant antibacterial potential in comparison to chitin. The enzyme chitinases can be utilized as the potential marker in the treatment of the fungal pathogenic diseases in plants and animals. Further the chitinases gene can be isolated and expressed in plants and animals for producing its expression and treatment of fungal diseases.
Table 1: Antibacterial activity of Chitinases and Chitin-based derivatives against pathogenic and drug resistant strains

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Standard Chitin</th>
<th>Extracted Chitin</th>
<th>Standard Chitosan</th>
<th>Extracted Chitosan</th>
<th>Standard Chito-oligosaccharide</th>
<th>Extracted Chito-oligosaccharide</th>
<th>Purified Chitinase enzyme</th>
<th>Chloramphenicol</th>
<th>Erythromycin</th>
<th>Negative Control (0.1% NaOH)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>13.0</td>
<td>13.0</td>
<td>14.0</td>
<td>18.0</td>
<td>16.0</td>
<td>19.0</td>
<td>11.0</td>
<td>39.0</td>
<td>25.0</td>
<td>NA</td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td>NA</td>
<td>12.0</td>
<td>10.0</td>
<td>14.0</td>
<td>11.0</td>
<td>12.0</td>
<td>NA</td>
<td>28.0</td>
<td>25.0</td>
<td>NA</td>
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<tr>
<td><em>S. abony</em></td>
<td>20.0</td>
<td>12.0</td>
<td>12.0</td>
<td>19.0</td>
<td>NA</td>
<td>NA</td>
<td>17.0</td>
<td>23.0</td>
<td>18.0</td>
<td>NA</td>
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<tr>
<td><em>Micrococcus</em></td>
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<td>NA</td>
<td>NA</td>
<td>19.0</td>
<td>18.0</td>
<td>19.0</td>
<td>21.0</td>
<td>39.0</td>
<td>33.0</td>
<td>NA</td>
</tr>
<tr>
<td><em>S. aureus 1</em></td>
<td>15.0</td>
<td>18.0</td>
<td>19.0</td>
<td>NA</td>
<td>23.0</td>
<td>NA</td>
<td>13.0</td>
<td>28.0</td>
<td>29.0</td>
<td>NA</td>
</tr>
<tr>
<td><em>S. aureus 2</em></td>
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<td>NA</td>
<td>10.0</td>
<td>12.0</td>
<td>19.0</td>
<td>21.0</td>
<td>20.0</td>
<td>30.0</td>
<td>20.0</td>
<td>NA</td>
</tr>
<tr>
<td><em>S. aureus 3</em></td>
<td>8.0</td>
<td>NA</td>
<td>13.0</td>
<td>13.0</td>
<td>NA</td>
<td>NA</td>
<td>10.0</td>
<td>11.0</td>
<td>30.0</td>
<td>26.0</td>
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<tr>
<td><em>Acinetobacter</em></td>
<td>18.0</td>
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<td>14.0</td>
<td>13.0</td>
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<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

*NA, No activity; S. aureus 1, 2, 3: Drug resistant Staphylococcus aureus isolated from different clinical samples*
Figure 1 (a): Antibacterial activity of Chitinases and Chitin-based derivatives against pathogenic and drug resistant strains.
CONCLUSION

The present study thus illustrates the antibacterial properties of Chitin and Chitin based derivatives extracted from the crustacean wastes. The study thus stresses the utilization of the crustacean waste as an important antimicrobial agent. Further studies on dose optimization and study of other pharmacological aspects may lead to the determination of fruitful and beneficial aspects of this wonder molecule.

REFERENCES


