Production of chitinase by using *Acremonium sporosulcatum* from shrimp biowaste

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Abstract:

Chitin is an essential structural component of the fungal cell wall and is present in the exoskeleton of arthropods and the microfilarial sheath of nematodes, acting as a protective layer against the harsh conditions that may be endured by the pathogen or arthropod. Mammals do not synthesize chitin, yet it is the second most abundant glycopolymer on earth, with an estimated 1010 tonnes of chitin produced each year. It was generally assumed that mammals lacked the ability to produce chitinase proteins, the enzymes responsible for chitin degradation. In the present study we aimed to produce chitinase enzyme by using *A. sporosulcatum* from shrimp biowaste. Chitinase activity was determined by a DiNitroSalicilic acid (DNS) method.

Introduction

Chitin, poly β-(1-4)-N-acetyl-D-glucosamine, is a natural polysaccharide of major importance, first identified in 1884. The importance of chitin was not realized for long time. The chitin is synthesized by enormous number of living organisms next to cellulose, chitin available abundantly in crystalline micro fibril form as a preventive mechanism. It has been as a part of exoskeleton of arthropods and in the cell walls of fungi and Yeast. It is also produced by a number of living organisms in the lower plant and animal kingdom, useful in many functions where reinforcement and strength are required.

Chitin occurs naturally as partially deacetylated form (with low content of glucosamine units) depending on its source; α and β chitins could not dissolved in usual solvents due to its distinguished crystalline nature. The insolubility is a major problem that confronts the development of processing and usage of chitin. Solid-state transformation of β chitin into α chitin occurs by treatment with strong aqueous HCL and washing with water. In addition, β form has found to be more reactive than the α form, a distinguished property of chitin with regard to enzymatic and chemical transformations.

Chitins are a large family of glycans that are β-1-4 linked, insoluble linear polymers of N-acetylglucosamine. They are present in the walls of higher fungi, exoskeleton of insects, arachnids,
invertebrates and as an extra cellular polymer of some bacteria. It is estimated that the rate of annual formation of chitin is on study state increase year after year. Bioaccumulation of non degradable chitin leads to great disaster in the world. Therefore, the application of thermo stable chitin-hydrolyzing enzymes (Chitinases) could be alternative for the effective utilization of this abundant biomass. In view of this, various chitinase genes and their phenotypic expressions from Eukaryotes and Bacteria have been well investigated (Patil. 1999).

Chitinases are digestive enzymes that break down glycosidic bonds in chitin.[1] Because chitin composes the cell walls of fungi and exoskeleton elements of some animals (including worms and arthropods), chitinases are generally found in organisms that either need to reshape their own chitin[2] or to dissolve and digest the chitin of fungi or animals. Chitinase is an extracellular enzyme which is capable in hydrolyzing insoluble chitin to its oligomeric and monomeric components. The enzyme produced by thermophilic bacteria was screened and isolated from Sulfili hot spring in Pinrang, South Sulawesi, Indonesia. The gram positive spore forming rod shape bacteria was identified as Bacillus sp. HSA,3-1a through morphological and physiological analysis. The production of chitinase enzyme was conducted at various concentration of colloidal chitin at a pH of 7.0 and a temperature of 55 °C. The bacteria optimally was produced the enzyme at a colloidal chitin concentration of 0.5% after 72 h of incubation. The optimum temperature, pH and substrate concentration of chitinase were 60 °C, 7.0 and 0.3%, respectively. The enzyme was stable at a pH of 7.0 and a temperature of 60 °C after 2 h of incubation. The chitinase activities was increased by addition of 1 mM Mg2+, Ca2+ and Mn2+ ions, whereas the activities were decreased by 1 mM Co2+, Fe2+ and Zn2+ ions. The molecular weight of the crude enzyme was determined using SDS-PAGE analysis. Five protein fractions were obtained from SDS-PAGE, with MWs of 79, 71, 48, 43 and 22 kDa Chitinivorous organisms include many bacteria (Aeromonads, Bacillus, Vibrio, [4] among others), which may be pathogenic or detrivorous. They may attack arthropods, zooplankton or fungi; or they may degrade the remains of these organisms. Fungi, such as Coccidioides immitis, are known to possess chitinases. This may be related to their typical role as detrivores and also to their potential as arthropod pathogens.

Production of chitinase: Single-parameter optimization was carried out for the production of chitinase using a soil isolate belonging to ‘Trichoderma harzianum’ in solid-state fermentation. Maximum chitinase activity (3.18 U/gds) was obtained after 96 h of incubation at 30 °C when wheat bran moistened (65.7%) with salt solution was supplemented with colloidal chitin (1%, w/w) and yeast extract (2%, w/w) used as the substrate. The inoculum contains 4×10⁷ spores of T. harzianum (TUBF 781). The growth of the fungus on wheat bran particle was visualized by a scanning electron microscope. Chitinase activity was measured as the amount of N-acetyl glucosamine (NAG) liberated in μmol/min under reaction conditions. The crude extract showed antifungal activity against a wide range of fungal strains belonging to Aspergillus, Rhizopus and Mucor sp., and was found significant against Aspergillus niger.

Objectives of the study

- Isolation of Microorganisms
- Collection of shrimp biowaste
- Optimization of sucrose
- Optimization of sodium nitrate
- Optimization of pH
- Optimization of temperature
- Optimization of different substrates

Materials and methods

Microorganism and inoculum preparation: A fungal isolate Acremonium sporsulcatum obtained from the HIB Nellore. This was used in the present study. The culture was maintained on nutrient agar medium and sub cultured every 30 days slants were prepared and incubated for two days at 30°C and stored at 4°C. The spores after fully sporulation, slants were dispersed in 10ml of 0.1% tween 80 solution by dislodging them with a sterile loop under aseptic conditions. The spore suspension obtained was used as inoculum viable spores present in the suspension were determined by serial dilution followed by plate count.

Shrimp biowaste: The waste was kindly provided by Shrimp care unit. The shrimp waste was washed with tap water then distilled water thereafter, exposed to water vapour and air dried at room temperature.

Chitinase assay: Chitinase activity was determined by a DiNitroSaliclic acid (DNS) method. This method works on the concentration of N-acetyl glucosamine which is released as a result of
enzymatic action. The 2ml reaction mixture contained 0.5ml of 0.5% colloidal chitin in phosphate buffer (pH-5.5), 0.5ml crude enzyme extract and 1ml distilled water. The well vortexed mixture was incubated in a orbital shaker at 50°C temperature for 1hr. The reaction was arrested by the addition of 3ml DNS reagent followed by heating at 100°C for 20mins. The absorption of colored solution was measured at 540nm using UV-spectrophotometer along with substrate and enzyme blanks. Colloidal chitin was prepared by the modified method of Robert’s and Selitrenkoff. One unit of the chitinase activity is defined as the amount of enzyme that is required to release 1µM of N-acetyl D-glucosamine per minute from 0.5% of dry colloidal chitin solution under assay conditions.

Preparation of n-acetyl d glucosamine standard curve:
100mg of was N-Acetyl D Glucose amine weighed. It was made up with 100ml of distilled water. Different concentrations of N-acetyl D glucose amine standard solutions were taken in different test tubes that are 0.2ml, 0.4ml, 0.6ml, 0.8ml, 1ml.

Preparation of dintro salicylic acid (DNS):
- Take 0.5 gm of DNS and chopped in a mortar pistle.
- And weigh 2 gm of NAOH, is dissolved in 50ml of distilled water.
- Then to chopped DNS add NAOH drop by drop, to avoid crystal like substances.
- And then weigh 30 gm of Sodium. Potassium tartarate and dissolved in 40 ml of distilled water.
- Then filter the DNS solution and add sodium potassium tartarate to it.make it 200ml with distilled water.

Composition of Czapec - dox medium (250 ml):
- Sucrose :7.5 grams
- NaNO:30.5 grams
- MgSO4 :0.125 grams
- Kcl :0.125 grams
- KH2PO4 :0.0875 grams
- Fe So4 :0.0025 grams
- Distilled water :250 ml

After this procedure the following steps have been performed

1. Preparation of colloidal chitin
2. Preparation of standard curve
3. Optimization of carbon sources
4. Optimization of nitrogen sources
5. Optimization of ph for maximum production of chitinase
6. Optimization of temperature for maximum production of chitinase
7. Production of chitinase by using different substrates

Results:
A graph was plotted between N-acetyl glucose amine and OD values and a straight line was obtained (Table -1 & Figure 1). By using the standard estabished protocols colloidal chitin was prepared and it was stored at 4˚c for the usage in the experiments.

Chitinase was produced in czapecox medium with Acremonium sporosulcatum: The maximum activity of chitinase was obtained after 72 hrs is 15.08µg at a concentration of 2000mgs (Table-2 & Fig-2). The minimum activity of chitinase was obtained after 24 hrs is 0µg at a concentration of 2000mgs (Table-2 & Fig-2).

The maximum activity of chitinase was obtained after 72 hrs is 12.43µg at a concentration of 50mg (Table-3 & Fig-3). The minimum activity of chitinase was obtained after 48 hrs is 0.87µg at a concentration of 100mg (Table-3 & Fig-3).

The maximum activity of chitinase was obtained after 72 hrs is 15.29µg at a pH 7 (Table-4 & Fig-4). The minimum activity of chitinase was obtained after 24 hrs is 0.25µg at a pH 8 (Table-4 & Fig-4).

The maximum activity of chitinase was obtained after 72 hrs is 10.04µg at room temperature (Table-5 & Fig-5). The minimum activity of chitinase was obtained after 24 hrs is 0µg at 4˚c (Table-5 & Fig-5).

The maximum activity of chitinase was obtained after 72 hrs is 15.02µg at a concentration of 1000mg from shrimp biowaste(Table-6 & Fig-6). The minimum activity of chitinase was obtained after 24 hrs is 1.36µg at a concentration of 1000mg (Table-6 & Fig-6).
TABLE-1: Preparation of n-acetyl glucosamine standard curve

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Conc of N-acetyl glucosamine(ml)</th>
<th>Distilled water(ml)</th>
<th>DNS(ml)</th>
<th>OD VALUES AT 540nm</th>
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<td>Blank</td>
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<tr>
<td>2</td>
<td>0.2</td>
<td>1.8</td>
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<td>0.4</td>
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<td>0.8</td>
</tr>
<tr>
<td>4</td>
<td>0.6</td>
<td>1.4</td>
<td>1</td>
<td>0.12</td>
</tr>
<tr>
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<td>0.8</td>
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<td>1</td>
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<td>6</td>
<td>1.0</td>
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<td>1</td>
<td>0.18</td>
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</table>

TABLE-2: Optimization of sucrose

<table>
<thead>
<tr>
<th>S.no</th>
<th>Conc of sucrose(gm)</th>
<th>Activity for 24hrs (ug/ml/min)</th>
<th>Activity for 48hrs (ug/ml/min)</th>
<th>Activity for 72hrs (ug/ml/min)</th>
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<tbody>
<tr>
<td>1</td>
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<td>1.5</td>
<td>0.12</td>
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<td>4</td>
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<td>0.0</td>
<td>11.30</td>
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<td>5</td>
<td>2.5</td>
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<td>12.05</td>
<td>13.81</td>
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Table-3: Optimization of sodium nitrite

<table>
<thead>
<tr>
<th>S.No</th>
<th>Conc of sodium nitrate(gm)</th>
<th>Activity for 24hrs (ug/ml/min)</th>
<th>Activity for 48hrs (ug/ml/min)</th>
<th>Activity for 72hrs (ug/ml/min)</th>
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<tbody>
<tr>
<td>1</td>
<td>Control</td>
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<td>6.96</td>
<td>5.47</td>
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<tr>
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<tr>
<td>3</td>
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<tr>
<td>5</td>
<td>0.20</td>
<td>6.21</td>
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<td>0.37</td>
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</table>
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Table-4: Optimization of pH

<table>
<thead>
<tr>
<th>S.No</th>
<th>Different pH</th>
<th>Activity for 24hrs (ug/ml/min)</th>
<th>Activity for 48hrs (ug/ml/min)</th>
<th>Activity for 72hrs (ug/ml/min)</th>
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</thead>
<tbody>
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<td>Control</td>
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<td>2</td>
<td>6</td>
<td>12.55</td>
<td>4.26</td>
<td>11.80</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
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<td>15.29</td>
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<td>8</td>
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<tr>
<td>5</td>
<td>9</td>
<td>0.87</td>
<td>2.88</td>
<td>7.03</td>
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</tbody>
</table>

Table-5: Optimization of temperature

<table>
<thead>
<tr>
<th>S.No</th>
<th>Different temperature</th>
<th>Activity for 24hrs (ug/ml/min)</th>
<th>Activity for 48hrs (ug/ml/min)</th>
<th>Activity for 72hrs (ug/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>3.01</td>
<td>7.40</td>
<td>10.04</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0.00</td>
<td>0.00</td>
<td>2.00</td>
</tr>
<tr>
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<td>25</td>
<td>4.26</td>
<td>6.78</td>
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<tr>
<td>4</td>
<td>35</td>
<td>1.25</td>
<td>4.26</td>
<td>5.63</td>
</tr>
<tr>
<td>5</td>
<td>45</td>
<td>4.77</td>
<td>6.65</td>
<td>9.29</td>
</tr>
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</table>

Table-6: Production of chitinase using different substrates

<table>
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<th>S.No</th>
<th>Substrate</th>
<th>Activity for 24hrs (ug/ml/min)</th>
<th>Activity for 48hrs (ug/ml/min)</th>
<th>Activity for 72hrs (ug/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Shrimp biowastes</td>
<td>1.86</td>
<td>11.98</td>
<td>15.02</td>
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<tr>
<td>2</td>
<td>Sucrose</td>
<td>1.36</td>
<td>10.44</td>
<td>11.80</td>
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<tr>
<td>3</td>
<td>Chitin</td>
<td>0.50</td>
<td>0.12</td>
<td>2.26</td>
</tr>
<tr>
<td>4</td>
<td>Colloidal chitin</td>
<td>0.37</td>
<td>0.24</td>
<td>2.76</td>
</tr>
</tbody>
</table>
Discussion:

Chitin is one of the most abundant biopolymers widely distributed in the marine and terrestrial environments. The role of the chitinolytic enzymes in nature is to degrade the huge quantities of chitin for recycling. Renewed commercial interest in the production and utilization of chitin and chitinolytic enzymes has demonstrated the need for inexpensive reliable sources of stable chitinase and chitobiase. Presently, commercially available purified and semipurified chitinases are obtained from microorganisms at a high cost, yet of unreliable specific activity. It is imperative to increase the supply of active chitinase while reducing the cost of production. This challenge may be met by extracting both chitinase and chitobiase from low cost readily available nonmicrobial sources (such as soybean seeds), and by recombinant DNA technology to develop chitinase/chitobiase - overproducing microbial strains. Furthermore, this paper will consider the important role of chitinases for the biological control of soil-borne plant pathogenic pests as well as biomass recovery from shellfish and cheese manufacturing industries.

It was generally assumed that mammals lacked the ability to produce chitinase proteins, the enzymes responsible for chitin degradation. However, recent findings have not only demonstrated that mammals produce chitinases, but also that increased secretion of chitinases is closely associated with T-helper type 2 (Th2)-dominated pathophysiological conditions including infection, fibrosis, allergy and asthma. Chitinases belong to the glycoside hydrolase family 18, which also encompasses enzymatically inactive chitinase like proteins (CLPs). Only the true chitinases have a functioning catalytic domain, which facilitates the hydrolysis of glycosidic bonds, resulting in chitin degradation.

While the role of chitinases and CLPs in settings of human allergic inflammation and other pathologies have only recently been highlighted, more information is available on the function and evolution of chitinases in other organisms. This review gives a broad perspective of the effects and functions of chitinases and CLPs in the context of their association with allergy and asthma.

Chitinases are digestive enzymes that break down glycosidic bonds in chitin. Because chitin composes the cell walls of fungi and exoskeletal elements of some animals (including worms and arthropods), chitinases are generally found in organisms that either need to reshape their own chitin or to dissolve and digest the chitin of fungi or animals. Chitinase is an extracellular enzyme which is capable in hydrolyzing insoluble chitin to its oligomeric and monomeric components.

Chitinase and chitin deacetylase are enzymes capable of degrading chitin into chitin oligomers and chitosan. The chitinases characterized and purified in this study were extracted from Acremonium sp. When grown in media containing colloidal chitin. The optimum chitinase activity of Acremonium was reached after 2-3 days of incubation. The optimum temperature and pH of the chitinase was found at 37°C and 7 and shrimp biowaste as substrate.

Conclusion:

Crustacean shells constitute the traditional and current commercial source of chitin. Conversely, the control of fungal fermentation processes to produce quality chitinase makes fungal mycelia an attractive alternative source. Therefore, the exploitation of both of these sources to produce chitinase in a concurrent process should be advantageous and is reported here. Acremonium sporusulcatum was selected from a screening for chitinase activity. The concurrent production of chitinase from shrimp shells and fungi by placing shrimp shells in direct contact with the fermentation of filamentous fungi was studied. The maximum production of chitinase was observed at room temperature and at pH 7 by taking shrimp biowaste as substrate. Chitinase is having wide industrial applications. Particular in the protease and other food industries use chitinase in vaste amounts. To meet the requirement an attempt to produce chitinase was tried in the present work.

Chitinase was produced by optimizing different carbon and nitrogen sources. Apart from the above sources chitinase was also produced using shrimp biowaste. The results have shown that chitinase can be effectively produced by using shrimbpbiowaste using Acremonium sporusulcatum.

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