Purification and characterization of chitinase from two Bacillus sp isolated from crustacean shells

V. Anuradha*1,2 and K. Revathi1

1Department of Zoology, Ethiraj College for Women, Chennai, India
2Department of Biochemistry, Mohamed Sathak College of Arts and Science, Sholinganallur, Chennai

ABSTRACT

Two Chitin degrading bacterial strains of Bacillus sp - Bacillus subtilis and Bacillus atrophaeus were isolated from crustacean shells. The strains were confirmed by Biochemical analysis, FAME-GC analysis and 16s rDNA sequencing. The chitinase enzyme was purified by a two step chromatographic method and characterized. The enzyme was purified to homogeneity by 6.75 fold with 46% recovery after ion exchange chromatography followed by gel filtration chromatography. The purified enzyme revealed a single band on SDS-PAGE gel with a molecular mass of 24 kDa. It showed an optimum pH at 6.0. The optimum temperature for enzyme activity was 40 °C. The maximum activity was observed with a 2% substrate concentration of colloidal chitin. The enzyme was strongly inhibited by Fe2+ and K+ while enhanced by Zn2+ and Ca2+. Thus the purification of microbial chitinase from shell waste could be effectively utilized for the manufacturing of many chitin derived products.

Keywords: Chitinase, Crustacean shells, Bacillus sp, FAME-GC, ion exchange chromatography, gel filtration chromatography, Characterisation.

INTRODUCTION

Chitins are a large family of glycans which are β-1,4-linked, insoluble linear polymers of N-acetylglucosamine (GlcNAc). They are present in the walls of higher fungi, in the exoskeletons of insects, arachnids, and many other groups of invertebrates, and as an extracellular polymer of some bacteria. Chitin is the second most abundant organic compound (after cellulose). It has been estimated that the annual formation rate and steady-state amount of chitin is on the order of 1010 to 1011 tons.

The capacity to degrade chitin is widespread among taxonomic groups of prokaryotes including the gliding bacteria, vibrios, Photobacterium spp., enteric bacteria, actinomycetes, bacilli, clostridia and archaea. Bacteria employ several proteins, including chitin-binding proteins, to degrade chitin, but the hydrolysis by chitinase is the key step in the solubilization and mineralization of chitin. The capacity to degrade chitin would seem to be an important attribute of marine bacteria given the presumed high input of detrimental chitin into the sea. The biodegradation of chitin requires the synergistic action of several hydrolytic enzymes for efficient and complete breakdown. The combined action of endochitinases (EC 3.2.1.14) and exochitinases [chitobiosidases and β-N-acetyl hexosaminidase (EC 3.2.1.82)] results in the degradation of chitin polymer into the soluble N-acetyl D-glucosamine [1]. Chitinases are produced by different microorganisms which generally present a wide multiplicity of enzymes that are mainly extracellular. They have received increased attention due to their wide range of biotechnological application. Commercial interest in the utilization of chitin and its derivatives has led to the need for inexpensive, reliable sources of active and stable chitinase preparations. 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.

Chitinases are isolated from variety of bacteria including *Bacillus* spp. and some of them are reported to produce multiple forms of chitinases with different molecular masses [2,3,4,5,6,7]. *B. cereus* UW85 has been proven as a reliable biocontrol agent of Phytophthora damping off and root rot of soybean [8] and capable of producing two antibiotics responsible for disease suppression [9]. The chitinases produced by a chitinolytic strain of *B. cereus* was analysed and found that this strain excreted two chitinases. One of them was partially purified and its encoding gene was cloned. In addition, this chitinase was characterized and investigated on its antifungal activity toward *Botrytis elliptica*, a fungal pathogen of lily leaf and blossom blight [10]. Structural analysis of *Bacillus circulans* WL-12 shows that it secretes at least six kinds of chitinases into the culture mediums [11]. Chitinases are produced by different micro-organisms which generally present a wide multiplicity of enzymes that are mainly extracellular. They have received increased attention due to their wide range of biotechnological applications, especially in the production of chitoooligosaccharides and N-acetyl D-glucosamine [12], biocontrol of pathogenic fungi [13,14], preparation of sphaeroplasts and protoplasts from yeast and fungal species [15,16] and bioconversion of chitin waste to single cell protein [17].

The isolation and phenotypic, as well as molecular genetic, characterization of new chitinase-deficient *Bacillus licheniformis* strains able to efficiently deproteinate shrimp shell waste, eventually resulting in chitin of superior quality, is also described[18]. *B. subtilis* W-118, which was isolated from the soil in Taiwan, extracellularly produces antifungal materials [19]. Except for a chitosanase from *B.subtilis* KH1, [20] and a chitosanase from a mutant of *B. subtilis* IMR-NK1, [21] specific features of the synthesis and secretion of chitinolytic enzymes by strains of Bacillus species have not been characterized in detail, unlike the case of actinomycetes and *Serratia marcescens*, which serve as sources of commercially available chitinases [22]. A number of chitin degrading bacteria were isolated from soil samples collected from agricultural fields of Tamil Nadu, India [23]. Chitinases from marine bacteria have been isolated and their properties reported. However, chitinases produced by marine bacteria isolated from coastal areas of Tamilnadu are not studied extensively. Hence, in this paper we describe the isolation of Chitin degrading bacterial strains and the characteristics of the chitinase produced them.

**MATERIALS AND METHODS**

1.1. Screening and isolation of Chitin degrading Bacteria

Exoskeletal bacterial isolates were obtained by abrasion of shell areas of both dorsal and ventral surfaces using a sterile scalpel. Each scraping was transferred into 600µl sterile 3.2%NaCl solution, homogenized briefly and spread-plated in triplicate onto MA and chitin agar [24]. After incubation for 5-7 days at room temperature, clearance zone forming bacteria were selected as the chitinase producer. Pure cultures from randomly chosen colonies were placed on MA slopes and stored at 4°C until use. Those strains were selected for the production and characterization of chitinase. The isolates were identified through a) it’s morphological and physiological properties according to Bergey’s manual of Systematic Bacteriology. b) The nearly complete nucleotide sequence of 16S rRNA was determined using bacterial universal primers. The 16S rRNA sequence was compared to the sequences in the GenBank nucleotide database by using Basic Local Alignment Search Tool (BLAST) and c) FAME-GC analysis.

1.2. Enzyme production and extraction

For enzyme preparation, the strains were grown at 28°C in colloidal chitin medium consisting of (g/l): colloidal chitin,10; peptone,3; KNO₃,3; KH₂PO₄,0.7; MgSO₄,0.5; KCl,1.0; and cultured in 50 ml medium in 250ml conical flasks with shaking at 180rpm for 3 days. The culture fluid was centrifuged at 8,000xg for 20 mins. Chitinase was extracted from the above bacterial bran by ammonium sulfate precipitation upto 75% saturation and the precipitate was resuspended in a minimum volume of 20mmol/l citrate phosphate buffer (pH 5.8). The precipitate obtained after ammonium sulfate fractionation was extensively dialysed against the same buffer for 24hrs at 4°C with continuous stirring and occasional changes of the buffer [25]. The resultant dialysate was chitinase crude extract and was subjected to further purification.

1.3. Purification of Chitinase

The dialysate obtained above was concentrated by lyophilisation and loaded on top of the DEAE-Sephadex A-50 (Pharmacia) column equilibrated with dialysis buffer. The procedure was carried out at 4°C. The concentrated active fractions from anion exchange chromatography were loaded onto a Sephadex G-100 column with 50 mmol/l
Sodium acetate buffer (pH 4.6) containing 0.1 mol/l NaCl. The enzyme was eluted in 2ml fractions with a linear gradient from 0.2 to 1.0 mol/l 20mM bis tris buffer (pH7.0) at a flow rate of 1ml/min in a sequential manner.

Each fractions were analysed for enzyme activity and protein content. The active fractions were pooled and concentrated by lyophilisation. The purified samples were stored at 20°C for further studies.

1.4. Chitinase assay
The strains were preinoculated in TSA for 24hrs and thereafter inoculated into minimal salt agar media and the activity was analysed every 24 hrs till it reaches a decline phase. The enzyme activity was also measured with crude culture supernatant, after ammonium sulfate precipitation and the pooled elutes obtained after purification steps (ion-exchange and gel filtration chromatography).

Chitinase activity was determined colorimetrically by detecting the amount of GlcNAc released from a colloidal chitin substrate [26]. The reaction mixture consisted of 0.3ml of crude enzyme and 0.2ml of colloidal chitin. The reaction was performed at 37°C for 30min. The mixture was boiled for 10min, chilled and centrifuged to remove insoluble chitin. The resulting adduct of reducing sugars was measured by DNA method [27]. GlcNac was used as the standard. One unit of Chitinase activity is defined as the amount of enzyme that released 1 µmol of GlcNAc from colloidal chitin per minute. The yield of the enzyme was also measured by calculating the specific activity and fold purification of the enzyme.

1.5. Protein estimation
The concentration of protein present in crude culture supernatant, after ammonium sulfate precipitation and the pooled elutes obtained after purification steps (ion-exchange and gel filtration chromatography) were determined using Bovine Serum Albumin (BSA) as the standard [28]. The reaction mixture consists of 0.2 to 1.0 ml of standard in 0.1N NaOH. The test solution contains 0.2 ml of each sample. To the solution, 5ml of CBB R250 was added and incubated for 10 minutes. The absorbance was measured colorimetrically at 595nm.

1.6. Characterization of the purified enzyme
Protein analysis was done by Sodium dodecyl sulfate (SDS) – polyacrylamide gel electrophoresis (PAGE) with 10% gels [29]. The pure protein sample from each of the bacterial isolates obtained after gel filtration was run along with standard molecular markers such as BSA 66kDa, Glutamate dehydrogenase 55kDa, Ovalbumin 45kDa, Carbonic anhydrase 30kDa and Trypsin 21kDa.

To determine the optimum pH of the enzyme, Chitinase activity was assayed at different pH values (pH 5.0 to 9.0) and Optimum temperature was measured by incubating the reaction mixtures at different temperatures to assay the enzyme activity. Chitinase activity was assayed at different temperatures ranging from 10-50°C at pH 5.5 in citrate phosphate buffer (50 mM). The effect of substrate concentration on chitinase activity was determined at different concentrations of chitin, varying between 0.5 mg ml-1 to 2.5 mg ml-1 (w/v). The effect of metal ions on enzyme activity was studied by incorporating these metal ions at 10 mM concentration such as KCl, CaCl₂,2H₂O, ZnSO₄ and FeSO₄ each at different volumes ranging from 0.2 – 1ml in reaction mixture.

RESULTS AND DISCUSSION

2.1. Screening and identification
In this present study Chitinase producing two bacterial strains were isolated from waste crustacean shells and they were identified as Bacillus subtilis and Bacillus atrophaeus based on their biochemical characteristics (Table 1), FAME-GC (Table 2) analysis and 16srRNA sequencing. The accession numbers of the 16srDNA is given (Table 3).

The physiological and biochemical characteristics of the two Bacillus strains were

i) Bacillus subtilis is a gram positive bacteria which shows positive results for gelatinase, catalase, Oxidase and fermentation of sucrose and mannitol. However, it shows negative for all the other tests like gelatinase, citrate utilization, indole production and sugar fermentation.

ii) Bacillus atrophaeus is a gram positive bacteria which shows positive results for Nitrate reductase, catalase and fermentation of all the sugars tested. However, it shows negative for all the other tests like gelatinase, Oxidase, citrate utilization and indole production.
Table 1. Biochemical Characterisation

<table>
<thead>
<tr>
<th>S.NO</th>
<th>Test</th>
<th>Bacillus subtilis</th>
<th>Bacillus atrophaeus</th>
</tr>
</thead>
<tbody>
<tr>
<td>01</td>
<td>Gram Staining</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>02</td>
<td>Spore formation</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>03</td>
<td>Nitrate reduction</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>04</td>
<td>Catalase</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>05</td>
<td>Oxidase</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>06</td>
<td>Gelatin hydrolysis</td>
<td>+ve</td>
<td>-ve</td>
</tr>
<tr>
<td>07</td>
<td>Citrate Utilisation</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>08</td>
<td>Methyl red</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>09</td>
<td>Indole production</td>
<td>-ve</td>
<td>-ve</td>
</tr>
<tr>
<td>10</td>
<td>Sugar fermentation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a)</td>
<td>Sucrose</td>
<td>+ve</td>
<td>+ve</td>
</tr>
<tr>
<td>b)</td>
<td>Arabinose</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>c)</td>
<td>Xylose</td>
<td>-ve</td>
<td>+ve</td>
</tr>
<tr>
<td>d)</td>
<td>Mannitol</td>
<td>+ve</td>
<td>+ve</td>
</tr>
</tbody>
</table>

Table 2. FAME-GC analysis of the isolates

<table>
<thead>
<tr>
<th>S. No</th>
<th>Name of the Isolate</th>
<th>Similarity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacillus subtilis</td>
<td>0.533</td>
</tr>
<tr>
<td>2</td>
<td>Bacillus atrophaeus</td>
<td>0.477</td>
</tr>
</tbody>
</table>

Table 3. Accession number of partial 16s rRNA sequence submitted in Genbank

<table>
<thead>
<tr>
<th>S. No</th>
<th>Organism Name</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bacillus subtilis</td>
<td>GU734768</td>
</tr>
<tr>
<td>2</td>
<td>Bacillus atrophaeus</td>
<td>GU734768</td>
</tr>
</tbody>
</table>

2.2. Enzyme Assay for maximum production

Previous reports have shown that species of Bacillus are known to produce chitinolytic enzymes [3, 30, 31, 32, 33]. Bacillus subtilis showed maximum activity on 3rd day [72 hours] of incubation whereas the Bacillus atrophaeus showed maximum activity on 4th day [72 hours] of incubation (Fig. 1). Bacteria produce several chitinases, probably to hydrolyze the diversity of chitins found in nature. Chitins can vary by the arrangement of N-acetylglucosamine strands, degree of deacetylation, and presence of cross-linked structural components, such as proteins and glucans.

2.3. Purification of Chitinase

Chitinase from both the strains were purified by Ammonium sulphate precipitation and dialysis followed by a two step chromatographic technique- ion exchange chromatography followed by gel filtration chromatography. The overall purification summary is presented in Table 4 and Table 5. For all the samples, the chitinase activity and protein concentration was determined for the crude supernatant and after ammonium sulfate fractionation. It is clear from the results that the specific activity and fold increased after each step of purification.

Fig. 1 Chitinase production by respective strains for an incubation period of 5 days

Available online at www.scholarsresearchlibrary.com
Table 4. Purification of chitinase from *B. Subtilis*

<table>
<thead>
<tr>
<th>S. No</th>
<th>Fractions</th>
<th>Protein (mg)</th>
<th>Total Activity (u)</th>
<th>Specific Activity</th>
<th>Yield (%)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Crude</td>
<td>0.40</td>
<td>198.9</td>
<td>497.28</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Ammonium sulfate fractionation</td>
<td>0.16</td>
<td>149.18</td>
<td>909.67</td>
<td>75.0</td>
<td>1.83</td>
</tr>
<tr>
<td>3</td>
<td>Ion exchange</td>
<td>0.08</td>
<td>115.36</td>
<td>1442.0</td>
<td>58.0</td>
<td>2.90</td>
</tr>
<tr>
<td>4</td>
<td>Gel filtration</td>
<td>0.05</td>
<td>78.1</td>
<td>1562.0</td>
<td>39.26</td>
<td>3.14</td>
</tr>
</tbody>
</table>

Table 5. Purification of chitinase from *B. Atrophaeus*

<table>
<thead>
<tr>
<th>S. No</th>
<th>Fractions</th>
<th>Protein (mg)</th>
<th>Total Activity (u)</th>
<th>Specific Activity</th>
<th>Yield (%)</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Crude</td>
<td>0.55</td>
<td>69.62</td>
<td>126.58</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Ammonium sulfate fractionation</td>
<td>0.14</td>
<td>64.50</td>
<td>460.71</td>
<td>92.6</td>
<td>3.63</td>
</tr>
<tr>
<td>3</td>
<td>Ion exchange</td>
<td>0.09</td>
<td>59.67</td>
<td>663.05</td>
<td>85.7</td>
<td>5.20</td>
</tr>
<tr>
<td>4</td>
<td>Gel filtration</td>
<td>0.05</td>
<td>43.36</td>
<td>867.20</td>
<td>62.3</td>
<td>6.85</td>
</tr>
</tbody>
</table>

The molecular weight of the pure chitinase was determined to be 29KDa for *B. Subtilis*, and 25KDa for *B. atrophaeus*. The molecular weights of microbial chitinases range from 20,000 to 120,000 with little consistency. The molecular weights of bacterial chitinases are around 60,000–110,000, while those of actinomycetes are usually 30,000 or less, and those of fungi are higher than 30,000. The results showed that the molecular weight of is approximately the same as other bacterial chitinases, such as the chitinase enzymes from *B. circulans* No. 4.1 [34], *B. licheniformis* X-7u [35] and *B. thuringiensis* subsp. pakistani [26].

2.4. Charaterization of Chitinase

The enzyme was active over a range of pH 5.0 – 8.0 with an optimum pH of 5.0 for chitinase from *B. Subtilis*, pH 8.0 for *B. atrophaeus* (Fig. 2). Other bacterial chitinase stable over broad pH range were pH 4.0 to 9.0 of *Aeromonas* sp. No. 10S-24 chitinase [36], pH 6.0 to 9.0 of *Pseudomonas aeruginosa* K-187 [37], pH 5.0 to 8.0 of *Aeromonas hydrophila* H2330 chitinase [38], pH 4.0 to 9.0 for *Vibrio* sp. [39], pH 6.8 to 8.0 of *Bacillus* sp. NCTU2 chitinase [3] and pH 4.0 to 8.5 of *Bacillus cereus* strain 65 [40].

![Fig. 2 Effect of pH on activity of Chitinase](image_url)

The effect of temperature on activity of the enzyme was determined by subjecting them to various temperatures ranging from 10°C to 50°C (Fig. 3). The results indicated that the temperature optima for this enzyme is 40°C for *B. Subtilis* and 50°C for *B. atrophaeus*. This was in accordance with other reports in literature such as *Enterobacter* sp. NRG4 [6], *Arthrobacter* sp. NHBN-10 [41], *Vibrio alginolyticus* TK-22 [42]. Chitinase from *Vibrio alginolyticus* TK-22 was stable at 40°C for 30 min [42] and purified chitinase of *Vibrio* sp. P-6-1 was stable at 40°C but completely inactivated at 55°C in 30 min [43].

The effect of substrate concentration on the activity of the enzyme was determined by assessing the enzyme activity at different concentration of substrate ranging from 0.5% to 2.5% of Colloidal chitin. Results suggested that 2.0% colloidal chitin has high specificity for chitinase from both the samples. Hence, 2% Colloidal chitin was the ideal substrate concentration for the maximum activity of Chitinase of both the *Bacillus* sp (Fig. 4).
Fig. 3 Effect of Temperature on activity of Chitinase

Fig. 4 Effect of Substrate concentration on activity of Chitinase

Fig. 5 Effect of various metal ions on activity of *B. subtilis* Chitinase

Fig. 6 Effect of metal ions on activity of *B. atrophaeus* Chitinase
Among the different salt ions analyzed at different concentration, the ZnSO₄ was the best activator followed by KCl and CaCl₂ whereas, FeSO₄ showed the most inhibitory activity on the Chitinase activity (Fig. 5 and Fig. 6). Activation of chitinase by Ca²⁺ or Mg²⁺ is rare and reported in few cases only. At 100 mM concentration Cu²⁺ and Ag⁺ completely inhibited chitinase activity when incubated at room temperature for 1 hr. In Pseudomonas aeruginosa, Mg²⁺ and Na⁺ were inhibitory while Cu²⁺ activated the chitinase by 50% [37]. Stimulatory effect of Ca²⁺ (30%) and Mn²⁺ (20%) at 1 mM concentration on Pseudomonas sp. YHS-A2 chitinase has been reported by [44].

**CONCLUSION**

The present study addressed the ability of the two local isolates of Bacillus sp. to produce the chitinase enzyme and their antifungal potential has already been reported. Chitinase thus offers tremendous industrial potential as biocontrol agent as well for treatment of chitinous wastes from sea-food industry. Utilization of chitinous waste as a substrate in SSF could be of great relevance for both economical and environmental aspects. Extensive literature is available on improving chitinase yield using immobilized as well as recombinant microorganisms.

**REFERENCES**


Available online at www.scholarsresearchlibrary.com

Available online at www.scholarsresearchlibrary.com