Characterization of chitinases from microorganisms isolated from Lonar lake

Vijay B Bansode and Shyam S Bajekal*
Postgraduate and Undergraduate Department of Microbiology, Yashwantrao Chavan College of Science
Vidyanagar, Karad 415 124, India

Chitinases, the enzymes that breakdown chitin—the second most abundant polysaccharide in nature—are known to have numerous uses. Alkaline chitinases, in particular are considered to have a greater potential in this respect. Fifteen chitinolytic microbial strains were isolated from the alkaline soil of Lonar lake in Buldhana district of Maharashtra. In this paper, the characters of chitinases produced by the five most potent isolates are presented. All the enzymes exhibited maximum activity in the neutral to alkaline range of pH (from 7.0 to 9.6) with a wide stability range from pH 4 to 11. The temperature optima for all the enzymes were slightly on the higher side, between 35 and 60°C, with a stability range from 25 to 60°C. All the enzymes showed a typical response to substrate concentration. None of the enzymes had any specific requirement for any particular metal ion, though a considerable stimulatory effect of Ca²⁺ was observed on all the enzymes. Minor effects of Cu²⁺, Mn²⁺ and Na¹⁺ were also observed on some of the enzymes.

Keywords: alkaline, biocontrol, chitinases, fungal protoplasting, seafood waste
IPC Code: Int. Cl. 8 A01N63/04

Introduction

Chitinases are a group of enzymes that decompose chitin, the second most abundant polymer in nature and the most abundant in the marine environment. They are produced by a diverse range of life forms, such as snails, crustaceans, insects, vertebrates, plants and microorganisms. Among the microorganisms, approximately 90 to 99 per cent of the chitinolytic populations are actinomycetes. Only a fraction of them are bacteria and less than one per cent are fungi.

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, agriculture and even in cosmetics. The enzymes themselves have been found to be antifungal and nematicidal agents for agricultural biocontrol methods.

The seafood industry is a major source of chitinous wastes. The recycling of which is extremely important to retain the carbon nitrogen balance in the ecosystem. Chitinases are considered to have the potential for use in the management of these wastes through the process of composting.

Chitinases with alkaline pH optima are reported to have greater applicability in their uses and those with greater thermo-stability were most suitable in the composting process. This study, thus, concentrated on such enzymes that are produced by microorganisms isolated from the lake in the Lonar crater, having extreme environment. Lonar lake, situated in Buldhana district of Maharashtra, is the only lake in the world formed by meteorite impact in basalt rock. The water of this lake and, consequently, the soil in its littoral zone are characterised by very highly alkaline pH of 8.0 to 9.0. The microorganisms in this environment would, therefore, be unique and their biochemical activities would also be expected to be so. Chitinases produced by microorganisms in this environment would, therefore, be adapted to the alkaline pH. Fifteen chitinase producing microorganisms were isolated from this environment and they were screened for chitinase production. Five of them—two species of *Streptomyces*, one of *Nocardia*, one of *Bacillus* and a fungal isolate were found to be the most potent producers.

In the present paper, we report the results of our preliminary study on the characteristics of chitinases produced by these five isolates.

Materials and Methods

Preparation of Colloidal and Swollen Chitin

Colloidal chitin was prepared from practical grade crab shell chitin (Loba Chemie) as described by Hsu.
and Lockwood. Swollen chitin was prepared from the same practical grade chitin by the method of Hackman described in Gohel et al.

Isolation and Selection of Chitin Utilizers

Isolation of chitin utilizers was done through enrichment in colloidal chitin agar medium of following composition (g L⁻¹): colloidal chitin (4), MgSO₄·7H₂O (0.5), K₂HPO₄ (0.7), KH₂PO₄ (0.3), FeSO₄·7H₂O (0.01), MnCl₂ (0.001) and agar (20); 0.03% NaCl, and 0.03 and 0.02% yeast extract. By visual analysis, well-separated colonies that showed a zone of clearance around them were transferred onto slants as pure cultures. Colonies showing large zones of clearance were selected as potent chitinase producers.

Production of Chitinases

Colloidal chitin broths (100 mL) in 250 mL capacity Erlenmeyer flasks were inoculated with 1.0 mL spore suspensions (adjusted to 1.0 OD₆₀₀) of the isolates and incubated in a rotary incubator (Lab Hosp) at 150 rpm and 37-40°C for 12 d. The culture broths were centrifuged at 8000×g for 20 min and the cell free supernatant saturated with ammonium sulphate to 60-70% levels and kept at 4°C overnight to extract the enzymes. The precipitates were dissolved in 50 mM phosphate buffer (pH 7.0) and dialysed against distilled water at 4°C overnight through a dialysis membrane (Hi Media dialysis membrane-70) with a molecular weight cut off at 12,000 Da. The stocks thus obtained were preserved at 0-4°C in PVC bottles.

Chitinase Assay and Characterization

The assay system of Monreal and Reese, estimating reducing sugars released by enzyme action, was adapted for the study. In 2.5 mL buffer, 2.5 mL 1% substrate (swollen chitin) was added, followed by 0.5 mL crude enzyme preparation. The tubes were incubated at appropriate temperature for 1 h. After which the reaction was stopped by adding 3.0 mL 10% dinitrosalicylic acid (DNSA), followed by heating in a boiling water bath. The coloured solution was then centrifuged at 8000×g for 5 min and the absorption of supernatant was measured at 540 nm wavelength in a Systronics spectrophotometer. The reducing sugar was estimated from a standard curve of glucose. One unit of enzyme is defined as the amount of enzyme that catalyses the release of 1 μM of reducing sugar per minute under assay condition.

Effect of pH on Enzyme Stability and Activity

Four different buffers of 50 mM strength, glycine-HCl (pH 2.2-3.4), acetate (pH 3.6-5.6), phosphate (pH 5.8-8.0) and glycine-NaOH (pH 8.6-11.0) were used to create pH values from 2.2-11.0 for this study.

The stability of enzyme at different pH was tested by keeping a suitable amount of enzyme in buffers of various pH values ranging from 2.2 to 11.0 at 37°C for 1 h, after which their residual activities were determined. pH values within the stability range of the enzymes (4.0-11.0) were created in the respective enzyme reaction systems and the effect on reaction velocity (μM/min of reducing sugars released) was analyzed after 1 h of reaction at 37°C, using appropriate substrate controls.

Effect of Temperature on Enzyme Stability and Activity

Thermal stability was investigated by incubating the enzymes in buffers of their optimum pH at temperatures 0, 15, 28, 37, 45, 60, 80 and 100°C for 1 h, after which those from higher temperatures were cooled rapidly by immersing in ice before the residual activities of each were measured at 37°C.

Appropriate buffers to maintain optimum pH of each enzyme were used in the system and the enzyme substrate reaction was carried out at the different temperatures within the stability range of the enzymes for 1 h, after which the velocity of the enzyme reaction was measured in each employing appropriate substrate controls.

Effect of EDTA and Metal Ions

One-millimole solutions of EDTA and the chloride salts of 10 metals, Na⁺, K⁺, Ag⁺, Ca²⁺, Cu²⁺, Hg²⁺, Mg²⁺, Mn²⁺, Zn²⁺, and Fe³⁺ were used in the study. Varying volumes of each solution were added into the assay systems to obtain different final concentrations from 0.2-2.0 mM for the reaction. The enzyme substrate reactions were allowed to proceed for 1 h at their respective optimum pH and temperature and the relative activities were calculated with respect to the controls where the reactions were carried out in the absence of any additive.

Effect of Substrate Concentration

Varying volumes of 1% swollen chitin were added in the assay systems of each enzyme and the reactions with fixed enzyme volumes (0.5 mL) were carried out
at their respective optimum pH and temperature for 1 h before velocities of each enzyme were measured.

**Results**

**Effect of pH on Enzyme Stability and Activity**

The effect of pH on the stability and activity of the chitinases from the three actinomycete isolates, two *Streptomyces* spp. (SB1 and VB3) and one *Nocardia* sp. (SB4) is shown in Figs 1-3. While SB4 and VB3 showed stability up to pH 10 with an optimum pH 9, SB1 showed a tolerance of up to just pH 8 with its optimum activity at pH 6. The *Bacillus* (SB5) chitinase showed a broad range of pH tolerance from 7 to 11 with maximum activity at pH 9 (Fig. 4), while the fungal chitinase (VB2) showed a broad range of pH tolerance from 4.0 to 9.0 and an optimum of pH 7 (Fig. 5). The optimum pH determined for each enzyme in this experiment was used in future experiments.

**Effect of Temperature on Enzyme Stability and Activity**

Observations noted in Figs 6-10 indicate the temperature stability of all the chitinases to be on the higher side, between 60 and 80°C. Among the chitinases from actinomycetes, it was observed that the enzymes from *Streptomyces* had their optimum temperature of activity at 37°C, while the *Nocardia* chitinase showed its maximum activity at 45°C. The *Bacillus* chitinase also had an optimum temperature of 37°C, whereas the fungal enzyme had between 50 and 60°C, which is the highest optimum temperature range among all the five isolates. The optimum temperature determined for each enzyme was used in subsequent studies.

**Effect of Metal Ions**

Only 5 of the 10 metals tested showed any significant stimulatory effect on the enzymes (Table 1). As expected, Hg^{2+} inhibited all enzymes at even the lowest concentration, while EDTA had no effect on any of the enzymes.

Calcium was the only metal showing a consistent stimulating effect on all the enzymes, resulting in a 2.5 to 3.4-fold increase in their activity. Copper also stimulates all the enzymes, though slightly (1.2-1.7×). Magnesium enhanced the enzyme activity of only VB2 and VB3 by 3.5 and 2.3 times, respectively, while it showed no significant effect on the other three enzymes. Manganese caused a 1.7 to 2.8-fold increase in the activity of four enzymes, SB1, SB5, VB2 and VB3. Sodium also showed stimulatory effect on all the enzymes, but the extent of stimulation range from 1.2 to 2.1×.

**Effect of Substrate Concentration**

All the enzymes showed a typical relationship with their substrate (Figs 11-13), in which all of them are saturated at low concentrations and reach to a maximum velocity fairly soon, which is indicative of a good catalytic efficiency.
Chitinases with alkaline pH optima and stability are considered to have a major potential in biological control of insect pests. The peritrophic gut lining of insects is chitinous and has an alkaline pH. Chitinases with a better stability and activity in these conditions can be used in synergism with other biocontrol agents. In the current study, four chitinases, isolated from Streptomyces, Nocardia, Bacillus and a fungal isolate (showing characters similar to members of genus Penicillium), are found to have their pH optima in the alkaline range. The enzymes from Bacillus and Streptomyces hold the most promise as they are stable up to pH 10 and are also the most efficient. Use of alkaline chitinases from S. albidoflavous, Nocardiopsis albus and Bacillus sp. for such purposes has earlier been reported. However, one of the enzymes in the current study (SB1) has its pH optima in the acidic range and could possibly be exploited for control of fungal plant pathogens and for fungal protoplasting as reported for similar enzymes earlier.
Table 1—Effect of metal ions and EDTA on activity of chitinases

<table>
<thead>
<tr>
<th>Component</th>
<th>SB1</th>
<th>SB4</th>
<th>SB5</th>
<th>VB2</th>
<th>VB3</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>EDTA</td>
<td>1.1</td>
<td>1.098</td>
<td>1.1</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Na⁺</td>
<td>1.2</td>
<td>1.5</td>
<td>2.1</td>
<td>2</td>
<td>1.5</td>
</tr>
<tr>
<td>K⁺</td>
<td>1</td>
<td>0.96</td>
<td>0.9</td>
<td>0.84</td>
<td>0.87</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>0.79</td>
<td>0.68</td>
<td>0.84</td>
<td>0.76</td>
<td>0.86</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>3.2</td>
<td>3.4</td>
<td>2.8</td>
<td>2.5</td>
<td>3.2</td>
</tr>
<tr>
<td>Cu²⁺</td>
<td>1.43</td>
<td>1.2</td>
<td>1.5</td>
<td>1.2</td>
<td>1.7</td>
</tr>
<tr>
<td>Hg²⁺</td>
<td>0.01</td>
<td>0.1</td>
<td>0.02</td>
<td>0.01</td>
<td>0</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>1</td>
<td>1.5</td>
<td>1</td>
<td>3.5</td>
<td>2.3</td>
</tr>
<tr>
<td>Mn²⁺</td>
<td>1.7</td>
<td>1.2</td>
<td>2.8</td>
<td>2.1</td>
<td>1.9</td>
</tr>
<tr>
<td>Zn²⁺</td>
<td>0.95</td>
<td>0.89</td>
<td>0.88</td>
<td>0.93</td>
<td>0.94</td>
</tr>
<tr>
<td>Fe³⁺</td>
<td>1.1</td>
<td>0.98</td>
<td>0.93</td>
<td>1</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Fig. 7—Effect of temperature on stability and activity of chitinase from actinomycete isolate SB4

Fig. 8—Effect of temperature on stability and activity of chitinase from actinomycete isolate VB3

Fig. 9—Effect of temperature on stability and activity of chitinase from bacterial isolate SB5

Fig. 10—Effect of temperature on stability and activity of chitinase from fungal isolate VB21

Fig. 11—Effect of substrate concentration on activity of chitinases from actinomycete isolates (SB1, SB4 & VB3)
Alkaline chitinases are also considered useful in management of chitinous wastes, such as those generated by seafood manufacturing industries. As the process applied here is one of composting, enzymes with stability at higher temperatures are considered more useful. The enzymes reported in this study are in consonance with earlier reports with optimum temperature around 45-55°C and retention of 50% activity (stability) at 60°C.

None of the enzymes showed requirement of any specific metal ions for their activity, though Ca²⁺ and Na⁺ did showed a consistent stimulatory effect on all enzymes. The other metals, Mg²⁺, Mn²⁺ and Cu²⁺ stimulated some enzymes. Ca²⁺ is a known stimulator of extracellular enzymes; especially helping them to withstand high temperatures and could be of use in composting of seafood waste.

Acknowledgement

The authors are thankful to the management and administration of Yashwantrao Chavan College of Science, Karad for providing the necessary facilities in the Microbiology Department for this work.

References


