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Purification and characterization of thermostable chitinase from a novel *S. maltophilia* strain

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ABSTRACT

Aims: The presents study examines the purification and characterization of a chitinase from *S. maltophilia SJ602* strain isolated from a soil sample collected from Jamia Hamdard, New Delhi.
Methodology and Results: The purification steps included chitin affinity using colloidal chitin as the affinity matrix and column chromatography using Sephadex G-100. The chitinase was purified to 66 fold having a yield of 17%. The molecular weight of the chitinase was found to be around 29 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The pH and temperature optima of the purified chitinase were found to be at pH 5.5 and 60 °C, respectively.
Conclusion, Significance and Impact of the study: Besides showing a significant yield, the enzyme has a high thermal stability which has its applicability in the recycling of chitin waste.

Keywords: Chitinase, *S. maltophilia,* chitin affinity, chitinase purification

INTRODUCTION

The word chitin comes from a Greek word “envelope” and came to light in 1811. It came to be known as a substance that occurs in mushrooms (Ruiz-Herrera, 1978). Chitin, the second most abundant polysaccharide in nature, is a linear β-1, 4 linked polymer of N-acetylglucosamine (Flach *et al.*, 1992). Chitin, a structural component of the fungal cell wall (Blumenthal and Roseman, 1957), is also the major constituent of arthropod exoskeletons, tendons, and the linings of their respiratory, excretory, and digestive systems (Clark and Smith, 1936 and Herring, 1979). It is found in outer skeleton of insects, fungi, yeasts, algae, crabs, shrimps, and lobsters, as well as in the internal structures of a number of invertebrates (Bhattacharya *et al.*, 2007). A report based on lectin binding, endo-chitinase binding and enzymatic degradation suggests that the *Paralipophrys trigloides* (fish) has chitinous epidermal cuticle (Wagner *et al.*, 1993). In contrast to cellulose, chitin, in addition to a carbon source, can provide nitrogen (6.89%) as well (C:N = 8:1) (Monreal and Reese, 1969) which makes it a useful chelating agent (Muzzarelli, 1973). Chitin and associated materials have an extensive usage in drug delivery, wound healing, dietary fibre and waste water treatment (Kadowaki *et al.*, 1997, Dixon, 1995, Muzzarelli, 1997, Muzzarelli, 1999, Flach *et al.*, 1992). Chitin is a white, hard, inelastic polysaccharide, and is a major contribution to pollution in coastal areas (Muzzarelli, 1973, and Zikakis, 1984).

Catabolism of chitin includes the initial cleavage of the chitin polymer by chitinases into chitin oligosaccharides and additional cleavage to N-acetylglucosamine, and monosaccharides by chitobiases (Suginta *et al.*, 2000). Chitinases (EC 3.2.1.14, also called chitodextrinase; 1,4-s-poly-N acetylglucosaminidase; poly-s-glucosaminidase; s-1,4-poly-N-acetyl glucosaminidase) are glycosyl hydrolases which catalyzes the hydrolytic cleavage of the s-1,4-linked polymer of N-acetyl-β-D-glucosamine (GlcNAc) of chitin. Chitinases are known to be produced by numerous organisms that include bacteria *Bacillus, Aeromonas, Alteromonas* (Tsujibo *et al.*, 1993), *Pseudomonas, Serratia, Vibrio, Streptomyces* (Blaak and Schrempf, 1995), and *Escherichia* (West and Colwell, 1984), fungi (*Trichoderma and Aspergillus*), invertebrates and vertebrates (Bhattacharya *et al.*, 2007). The size range of chitinases varies from 20 kDa to about 90 kDa. Bacterial chitinases have a molecular weight range of ~20-60 kDa, which is similar to that of plant chitinases (~25-40 kDa) but are smaller than insect chitinases (~40-85 kDa) (Bhattacharya *et al.*, 2007). Chitinases have been divided into two main groups: endo-chitinases (E.C 3.2.1.14) and exo-chitinases (E.C 3.2.1.52). Comparative to exo-chitinases, endochitinases randomly split chitin at internal sites, thereby forming the dimmers of di-acetylchitobiose and soluble low molecular mass multimers of GlcNAc such as chitotriose, and

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chitotetraose (Sahai and Manocha, 1993). Chitinases hold a lot of importance due to the fact that they have vast bioprocessing and biotechnological aspects. They find use in things like biological control (Mathivanan *et al.*, 1998), production of ophthalmic products (Dahiya *et al.*, 2006), regeneration of protoplasts (Dahiya *et al.*, 2007), mosquito control (Mendonsa *et al.*, 1996) and production of single cell protein (Vyas and Deshpande, 1991), etc.

The objective of the current study was to isolate and purify the chitinase from a novel *S. maltophilia* strain followed by its characterization on the basis of pH, temperature, molecular weight, and chitinase activity. The purified chitinase was then subjected to different metal ions concentration for analysing their effect on chitinase activity.

MATERIALS AND METHODS

Bacterial Culture and Growth Conditions

The bacterial strain used in the present study was *Stenotrophomonas maltophilia SJ602* (Accession No. EU492391). It was isolated from the soil and later characterized by 16S rRNA sequencing of the amplified product (Khan *et al.*, 2010). The bacterial culture was inoculated in media containing chitin (4.94 g/l), maltose (5.56 g/l), yeast extract (0.62 g/l), KH_2PO_4 (1.33 g/l), and $MgSO_4 \cdot 7H_2O$ (0.65 g/l) as described by Khan *et al.*, (2010) and the pH was adjusted to 7.0. The bacterial culture was incubated at 37 °C for 72 h at an rpm of 180. The obtained culture was centrifuged for 20 min at 7,000 × g. The culture supernatant was filtered while the pellet was discarded. The supernatant was stored at 4 °C for further purification of the chitinase. Chitinase activity was estimated at each purification step.

Preparation of Colloidal Chitin

Colloidal chitin was prepared as described by Khan *et al.*, (2010). The practical grade chitin powder (HiMedia, India) was used to prepare the colloidal chitin. Chitin powder (40 g) was dissolved in 500 mL of concentrated hydrochloric acid and continuously stirred at 4 °C for 1 h. After stirring, the hydrolyzed chitin was washed a number of times with distilled water in order to remove the acid completely and hence bring the pH in the range of 6-7. As the desired pH was attained, the colloidal chitin was filtered through Whatman filter paper No.1. The sieved colloidal chitin was subsequently collected and stored in the form of a paste at 4 °C. This colloidal chitin was used at 5% of the composition of the medium as the sole carbon source with other minimal salts and agar.

Biochemical Quantification of Chitinase

The chitinase activity in the culture supernatant and at all purification steps was estimated as described by Khan *et al.* (2010) using colloidal chitin as the substrate (Vyas and Deshpande, 1991). The assay mixture for the chitinase activity contained 1 mL 5% colloidal chitin, 1 mL 50 mM acetate buffer, pH 5.0, and 1 mL enzyme solution, was incubated for 1 h at 50 °C. The reaction mixture was boiled for 15 min to stop the reaction followed by centrifugation for 20 min at 7,000 × g. The chitinase activity was measured spectrophotometrically (Spectronic 20 Genesys) at 540 nm based on the concentration of released GlcNac (N-acetyl D-glucosamine; the repeating units of chitin) produced with colloidal chitin as substrate (Monreal and Reese, 1969), from the aliquots following a 3, 5-Dinitrosalicylic acid (DNS) sugar estimation test using GlcNac as standard. One unit of chitinase activity was defined as the amount of enzyme, which releases 1 mM N-acetyl-D-glucosamine per minute under the standard conditions of this study.

Purification of Chitinase

All purification steps of chitinase were performed at 4 °C unless otherwise mentioned. The first step of the chitinase purification was chitin affinity. An equal volume of culture filtrate and 5% colloidal chitin was incubated overnight at 4 °C followed by centrifugation at 10,000 × g for 25 min, in order to remove the unabsorbed proteins. The supernatant was discarded and pellet was washed 2-3 times with an equal volume of 50 mM sodium acetate buffer (pH 5). The pellet was then dissolved in the same buffer and incubated for 3-5 h at 37 °C with continuous shaking for the release of enzyme from chitin. The enzyme/colloidal chitin suspension was then centrifuged at 10,000 × g for 25 min to eliminate the colloidal chitin in the form of pellet. The obtained clear supernatant was passed through an amicon ultrafiltration membrane with 10kDa cut off and afterwards applied to a Sephadex G-100 column which was pre-equilibrated with 50 mM Tris HCl (pH 7.5) containing 100 mM NaCl at a flow rate of 0.5 mL/min. The eluate was subsequently dialyzed overnight. The enzyme solution thus obtained was used for further characterisation of the chitinase.

Determination of Protein Concentration

In case of the crude samples protein content was determined by the method described by Lowry *et al.*, (1951) with BSA as standard.

Molecular Weight Determination

The molecular weight of the purified enzyme was determined under both native and denatured conditions by the method described by Laemmli (1970), using 12% resolving gel and 5% stacking gel. Silver staining was performed for the visualisation of protein bands. A broad range molecular weight marker (Merck, India) was used having myosin: 205 kDa; phosphorylase B: 97.4 kDa; bovine serum albumin: 66 kDa; egg albumin: 43 kDa; carbonic anhydrase: 29 kDa; lysozyme: 14.3 kDa; aprotinin: 3 kDa; insulin α and β chains 2.3-3.4 kDa.

Characterisation of Purified Chitinase

The purified enzyme was characterised on the basis of pH and temperature. The optimum pH was determined by

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varying the pH of the assay mixture between 3.0 and 9.5 at an increment of 0.5 pH unit. The temperature optimum of the purified enzyme was also determined with a temperature range of 10-80 °C. The buffer used was 50 mM sodium acetate.

The effect of different metal ions on the activity of the chitinase was also determined. The chitinase activity was assayed at 50 °C with an incubation period of 1 h. The metal ions used in the study were Fe^{2+}, Mg^{2+}, $Mn^{2+}$and Co^{2+}. The metal ions were used in three different concentrations of 2.0, 5.0 and 10 mM, respectively. The solution mixture having no metals (0 mM) was treated as control.

RESULTS and DISCUSSION

The summary of the purification of chitinase from novel *S. maltophilia* strain is given in the Table 1. The chitinase was purified upto 66 fold in a two step procedure with a recovery of 17%. The chitinase was purified to homogeneity which showed a single protein band on 12% SDS as well as on native PAGE. Its molecular weight was predicted to be approximately 29 kDa by SDS-PAGE (Figure 1). As reported, chitinases have been isolated from *Aeromonas* (Wu et al, 2001), *Bacillus* (Bhushan and Hoondal, 1998, Wen *et al.*, 2002), *Pseudomonas* (Lee *et al.*, 2000), *Serratia* (Duzhak, 2002) and *Streptomyces spp.* (Tanabe *et al.*, 2000) having a molecular weight range of 21 to 89.8 kDa. Few authors have reported about bacteria producing many types of chitinases differing in size while some produce only single chitinase such as Duzhak *et al.*, (2002) have reported the production of several chitinases with molecular weight of 62, 54, 43, 38 and 21 kDa by *Serratia marcescens*, etc. Researchers have also reported the production of several chitinases from *Bacillus*

In order to determine the optimum temperature of the purified chitinase, the reaction was carried out at the temperature range of 10-80 °C in 50 mM sodium acetate buffer (pH 5.5) using colloidal chitin as the substrate for 1 h. The chitinase exhibited maximum activity at 60 °C although it lost half of its activity at 70 °C. There was a substantial decrease in the chitinase activity after 60 °C and activity was negligible at 80 °C (Figure 3). The study of 29 kDa chitinase categorized here has optimal activity at pH 5.5 and at 60 °C was in close agreement with the bacterium *thuringiensis* subsp. *pakistani* having molecular weight of 66, 60, 47 and 32 kDa in size (Thamthiankul, 2001).

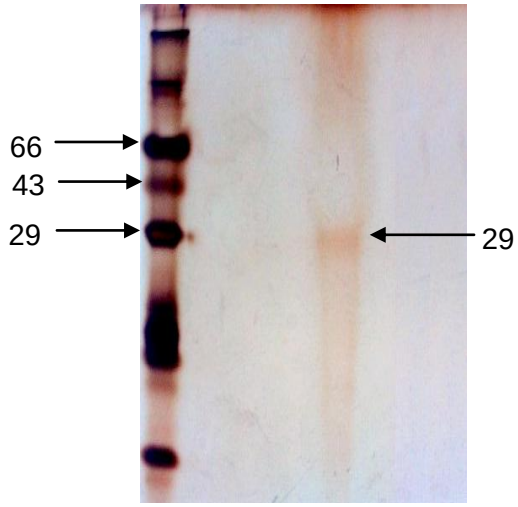

Figure 1: SDS-PAGE of the purified chitinase of bacterium *S. maltophilia* SJ602

Table 1: Summary of the chitinase purification.

Purification Steps	Total Activity Units (U)	Specific Activity (U/mg protein)	Fold Purification	Yield (%)
Crude	**2100**	**7**	**-**	**100**
Chitin affinity	**1250**	**20**	**2.88**	**59**
Ultrafiltration	**600**	**80**	**11.4**	**28**
Sephadex G-100	**375**	**468**	**66.9**	**17.85**

The pH optima studies on the purified chitinase showed that the enzyme was optimally active at pH 5.5. It showed a relatively good stability between pH 5-8 while at pH 9.5, activity of chitinase was almost lost (Figure 2). The pH optima studies of various other chitinases have shown that the chitinase from *T. lanuginosus* exhibited the highest activity at pH 4.5 (Guo *et al.*, 2008), *Aeromonas sp*. and *Ralstonia sp.* at pH 5.0. (Mitsuhiro *et al.*, 2005; Lien *et al.*, 2007), *Microbispora sp.* at pH 3.0. (Nawani *et al.*, 2002), *Bacillus cereus* at pH 5.8 (Wang *et al.*, 2001), while *Bacillus circulans* and *Beauveria bassiana* showed pH optima at pH 8.0 and 9.2 (Suresh and Chandrasekaran, 1999) respectively.

Microbispora sp. V2 (Nawani *et al.*, 2002) which produces chitinase at high temperature and acidic pH for optimal activity. High temperature optima have been reported for chitinases of *Bacillus licheniformis* (Takayanagi *et al.*, 1991), which produces four exo-chitinases with temperature optima of 70–80 °C and pH optima ranging 5.0–6.0. However, Sakai *et al.*, (1998) isolated three thermostable endo-chitinases at temperatures ranging from 65–75 °C Bacillus sp.

The effects of various metal ions on the chitinase activity are shown in Figure 4. It was found that the enzyme activity of chitinase was significantly increased with the addition of Mn^{2+} and Co^{2+}. From the fig., it was evident that chitinase enzyme was almost twice stimulated

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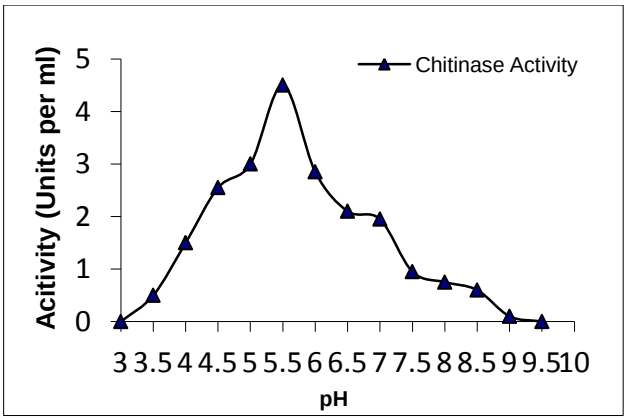

Figure 2: Effect of pH on activity of chitinase (—▲—) from bacterium *S. maltophilia* SJ602.

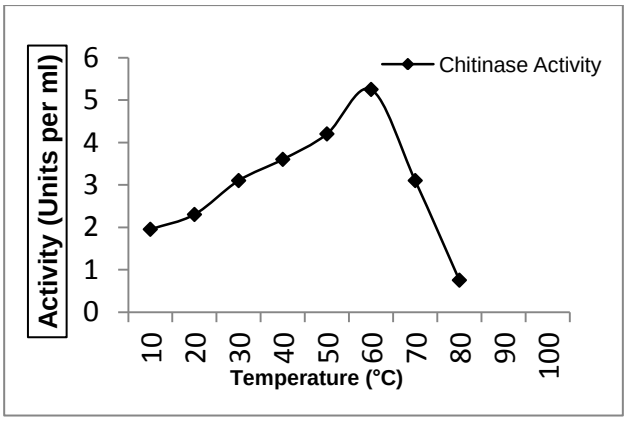

Figure 3: Effect of temperature on activity of chitinase (—▲—) from bacterium *S. maltophilia* SJ602

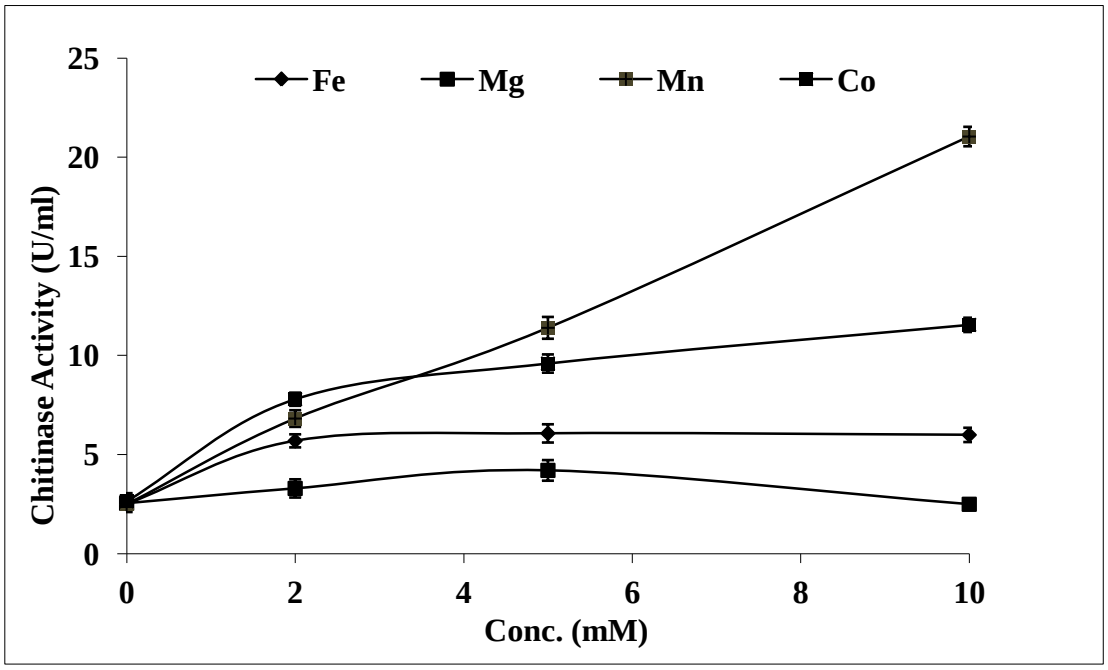

Figure 4: Effect of different metal ions on activity of chitinase from bacterium *S. maltophilia* SJ602 (a) Fe (—◆—), (b) Mg (—●—), (c) Mn (—■—), (d) Co (—✳—)

by Mn^{2+} ions than by Co^{2+} ions. The presence of Fe^{2+} ions showed slight stimulation in enzyme activity followed by stationary phase even after increasing the metal ion concentration. While, Mg^{2+} ions increased the chitinase activity at low concentrations, higher concentrations (5, 10 mM) showed decrease in the activity.

The effect of metal ions shows diversity on chitinase activity. Sakai *et al.*, (1998) however, reported that Fe^{2+} salts have inhibitory effect while Mg and Ca have stimulatory affect. This is in contrast to our results which might be due to the bacterial difference and geographical isolation. The chitinase from *Bacillus* sp. DAU101 (Lee *et al.*, 2000) and Indonesian *Bacillus* K29-14 (Sri *et al.*, 2004) were increased by Co^{2+}, while the chitinases from bacterium C4 (Yong *et al.*, 2004) and *Bacillus* MH-1 (Sakai *et al.*, 1998) were activated by Mn^{2+}. It was believed that, the thermostable chitinase from the thermophilic *Bacillus* sp. Hu1 appeared to be another different type of chitinase, since it was only slightly inhibited by Cu^{2+} ion and showed increased activity by Mg^{2+}, Ca^{2+} and Zn^{2+}.

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CONCLUSION

In this study, the purification and biochemical characterization of the chitinase produced by novel strain of S. maltophilia in a chitin medium was carried out and the results obtained showed significant yield of the enzyme by the method used. The use of colloidal chitin prepared from practical grade chitin makes it very cost effective for the production of chitinases.

This study also concludes that colloidal chitin can be repeatedly used for at least 10 times with the same sample or the different samples for chitinase production. The high thermal stability of the *S. maltophilia* SJ602 chitinase is predominantly beneficial for its applicability to the recycling of chitin wastes. Generally, during bioconversion of wastes temperature raises, and as the chitinase reported here have high thermal stability; it could be very useful at this stage of recycling. Conclusively, *Stenotrophomonas* sp. could be used as a suitable model

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