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Purification and characterisation of an acidic and antifungal chitin[illegible] produced by a *Streptomyces* sp.

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HIGHLIGHTS

- Purification of chitinase enzyme produced by a *Streptomyces* sp. was carried out.
- The molecular weight and the isoelectric point of the chitinase was determined.
- Characterisation of the enzyme was carried out with respect to pH, temperature a[illegible]sence of met[illegible]s and chemicals.
- The kinetic parameters of the enzyme were studied.
- The antifungal activity of the enzyme was studied.

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ABSTRACT

An extremely acid[illegible]lar chitinase produced by a *Streptomyces* sp. was purified 12.44-fold by ammonium sulphat[illegible]ecipi[illegible]-exchange chromatography and gel-permeation chromatography and further characte[illegible]d. [illegible] mo[illegible]ular mass of the enzyme was estimated to be about 40 kDa by SDS–PA[illegible]e optim[illegible] and temperature of the purified enzyme were pH 2 and 6, and 50 °C respectively. [illegible]me sho[illegible] high stability in the acidic pH range of 2–6 and temperature stability of up to 5[illegible] Additi[illegible]lly, the [illegible]t of some cations and other chemical compounds on the chitinase activity [illegible] studie[illegible]ctivity of the enzyme was considerably retained under salinity conditions of up to 3% [illegible] values of the enzyme were determined to be 6.74 mg mL^{-1} and 61.3 U mg^{-1} respectively [illegible] colloidal chitin. This enzyme exhibited antifungal activity against phytopathogens revealing a poten[illegible]iocontrol application in agriculture.

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1. Introduction

Chitin is [illegible]-1,4-[illegible]ty[illegible]cosamine (NAG) and is the next most [illegible]ndan[illegible]olysacc[illegible] found in nature after cellulose and is b[illegible]mpati[illegible] biodegradable and bioabsorbable (Khoushab and Yama[illegible]). [illegible]e principal component of arthropod exoskeletons [illegible] connective tissues, fungal cell walls and is also found to a lim[illegible] extent in other marine organisms. Among the best-characterise[illegible]urces of chitin are shellfish (including shrimp, crab, lobster, and krill), oyster and squid. The chitin contents in crustaceans usually range from 2% to 12% of the whole body mass while it constitutes of 22–40% of fungal cell walls. Up to 20–58% dry weight of waste generated from processing of shellfish consists of chitin (Dahiya et al., 2006; Gohel et al., 2006). In the natural state, chitin is found to be complexed with proteins, lipids, pigments and minerals such as calcium carbonate. Hence, demineralisation and deproteinization of the chitin containing biomass is necessary to obtain chitin in the pure form. Traditionally this is carried out using concentrated acids or alkalis, which poses corrosion, disposal and safety related environmental problems (Gohel et al., 2006). The alternative to this is processing of the chitin wastes using low-cost chitin degrading enzymes. Chitinases (EC.3.2.14) are chitin hydrolysing enzymes that have gained interest in different biotechnological applications due to their ability to degrade chitin in the fungal cell wall and insect exoskeleton. Based on the chitin hydrolysing characteristics, chitinases are classified into two types, exochitinase and endochitinase. Endochitinases cleaved randomly within the chitin polymer and cut them into shorter fragments while exochitinases or chitobiosidase (EC 3.2.1.29), hydrolyze chitin from the terminal end and release chitobiose. A third enzyme namely 1,4-β-*N*-acetylglucosaminidases (EC 3.2.1.30) hydrolyzes short oligomers, typically chitobiose dimer units, and releases *N*-acetylglucosamine (Hamid

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et al., 2013). The production of inexpensive chitinolytic enzymes is an important factor in the use of chitinous wastes, which would not only solve environmental problems but also offers other applications including their use as antimicrobial or insecticidal agents which are used in biocontrol of plant pathogens. Another useful application of chitinase is for bioconversion of chitin, into pharmacological active products, namely *N*-acetylglucosamine and chito-oligosaccharides. These are useful as antimicrobials, immuno-enhancers and activation of host defence system, drug delivery carriers, antioxidants, in haemostasis and wound healing, blood cholesterol control and food preservation (Khoushab and Yamabhai, 2010; Dahiya et al., 2006). Other interesting applications include the preparation of protoplasts from filamentous fungi and the production of single cell protein (Dahiya et al., 2006).

Chitinases are present in a broad range of organisms including viruses, bacteria, fungi, insects, higher plants and mammals (Gohel et al., 2006; Khoushab and Yamabhai, 2010). Most organisms (bacteria, plants and insects) have large families of chitinases with distinct functions, including digestion, defence against pathogens, cuticle turnover, and cell differentiation (Gohel et al., 2006; Khoushab and Yamabhai, 2010). Chitin is a high molecular weight complex polymer and is generally insoluble in aqueous media and hence its uptake as such is not possible. Hence bacteria, especially *Streptomyces* (Brzezinska et al., 2013; Margino et al., 2010; Nagpure and Gupta, 2013; Prakash et al., 2013; Pradeep et al., 2014) and *Bacillus* (Kudan and Pichyangkura, 2009; Liu et al., 2010; Natsir et al., 2010; Waghmare and Ghosh, 2010; Dai et al., 2011) species, produce extracellular chitinases for nutritional purposes, in that they are able to utilise chitin as their sole source of carbon. Application of chemical based pesticides causes long-l[illegible]ing adverse effects on ecosystems and human health. Hence, [illegible] necessary for searching and developing non-hazardous biologica[illegible] compatible alternatives. Such eco-friendly substitute of chemica[illegible] pesticides is considered as biocontrol agents. Inhib[illegible] killing of harmful pests by biocontrol agents is biolog[illegible]y sa[illegible] target specific and without creating any envir[illegible]ental [illegible]lution. Diseases caused by phytopathogenic fungi not [illegible] cant economic impacts in the agricult[illegible] indus[illegible]ut also on human well being and biodiversity. Th[illegible]ngal cell w[illegible]nd structural membranes of mycelia, stalk[illegible]ores contai[illegible]itin as the major component. Hence bacte[illegible]al chi[illegible]es that can degrade chitin are used as an effective [illegible]ontrol reme[illegible]gainst many fungal pathogens especially th[illegible] of plants (Gohel [illegible]., 2006). These chitinases are involved [illegible]e pro[illegible] of cell wall lysis and hence concentrated efforts are [illegible]e t[illegible]rify and characterise antifungal chitinases. In this study, a[illegible]mpt ha[illegible]en done to isolate and purify the aci[illegible]inase [illegible]th [illegible]fungal activity from a *Streptomyces* [illegible]ecies. [illegible] additi[illegible]rther characterization with respect to [illegible]etic an[illegible]sis of the purified chitinase has also been done.

2. Methods

2.1. Preparation of colloidal chitin

The colloidal chitin was prepared by the method of Roberts and Selitrennikoff (1988) with minor modifications. The colloidal chitin thus prepared was then stored at 4 °C for subsequent use.

2.2. Chitinase assay

Extracellular chitinase activity was determined by incubating 0.5 mL of crude enzyme with 1 mL 0.1 M citrate–phosphate buffer, pH 5.5 and 0.5 mL of 1% colloidal chitin (final colloidal chitin concentration at 0.25%) in the same buffer at 50 °C for 1 h. The mixture was kept in a water bath at 50 °C for 1 h along with appropriate blanks. The reaction was arrested by addition of 3.0 mL 3, 5 dinitrosalicylic acid (DNS) reagent (Miller, 1959), followed by heating for 10 min in a boiling water bath. After cooling to room temperature (30 °C), the coloured solution was centrifuged at 10,000×g for 5 min at room temperature and absorption of the supernatant was measured at 540 nm along against the control (blank) using a UV–Visible spectrophotometer (Shimadzu). One unit of the enzyme activity was defined as the amount of enzyme that catalysed the release of 1 μmol mL^{-1} min^{-1} of reducing sugar under the above mentioned assay conditions. The assay procedure was suitably modified according to th[illegible]ements of the experiments.

2.3. Microbial culture and chitinas[illegible] prod[illegible]n conditi[illegible]

A *Streptomyces* sp. was [illegible]iously isolate[illegible]dentified, and the bioprocess paramete[illegible]or ma[illegible]um chiti[illegible]e production by the same were optimised [illegible]et al., 2[illegible]13). The culture was inoculated in a chi[illegible] yeast [illegible]ct-salt[illegible]YS) medium with the following compo[illegible]on (g L^{-1}): [illegible]id[illegible]hitin, 14.0; glucose, 2.0; yeast extrac[illegible]NaCl, 2.0; [illegible]HPO_4, 0.8; KH_2PO_4, 0.4; $MgSO_4{\cdot}7H_2O$, [illegible].5; Ca[illegible]H_2O, 0.5 and pH 8.0. It was then incubated at 30 °C [illegible] 200 rpm sh[illegible]. After 60 h of incubation, the culture broth [illegible]rvested, centri[illegible]ation was done at 9500*g* for 10 min at [illegible] and the supernatant that consisted of the crude chitinase wa[illegible]rther used [illegible] enzyme studies.

2.4. E[illegible]e puri[illegible]tion and determination of molecular weight

[illegible]urification of chitinase enzyme was carried out using the f[illegible] methods. It was first purified by ammonium sulphate pre[illegible]pitation method, followed DEAE anion-exchange chromatography and further by gel permeation chromatography as shown in Table 1. 200 mL cell-free supernatant was precipitated with ammonium sulphate (60%). The pellets obtained after centrifugation at 9500*g* for 15 min at 4 °C were dissolved in citrate–phosphate buffer (0.1 M, pH 5.5) and dialyzed overnight in the same buffer. Ion-exchange chromatography was done using a DEAE-methyl acrylate copolymer (Toyopearl DEAE-650, Tosoh Bioscience, Japan) column (30 cm × 1 cm). The column was packed with overnight-swollen resin in 0.1 M citrate buffer (pH 5.5) and eluted stepwise with 0.1–0.8 M NaCl at a flow rate of 1 mL min^{-1}. The protein fractions (1 mL) were analysed for chitinase activity and those with positive activity was pooled and concentrated. This was then further applied to a gel permeation chromatographic (GPC) column (50 cm × 1.7 cm). The column was packed with overnight-swollen resin (Toyopearl HW50, Tosoh Bioscience, Japan) in 0.1 M citrate buffer (pH 5.5). The same buffer was passed through the column at a flow rate of 1 mL min^{-1} to separate the proteins. The protein fractions (1 mL) were analysed for chitinase activity. The total protein content was determined for all the fractions by Bradford's method using bovine serum albumin as the standard protein (Bradford, 1976).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS–PAGE) was carried out according to Laemmli (1970) using vertical slab gel electrophoresis unit. A 14% separating and 5% stacking gel were used for PAGE analysis. The molecular mass of purified enzyme was determined by using protein molecular mass-standards (Precision-Plus) obtained from Biorad, USA. After SDS–PAGE, gel was stained by silver staining method. The chitinase purified by DEAE-anion exchange chromatography was used for further characterisation studies including enzyme properties, antifungal activity and kinetics.

Gel permeation chromatography was used for determining the molecular mass of the chitinase. The separation was carried out

Table 1
Purification of *Streptomyces* sp. chitinase.

Purification Step	Volume (mL)	Total proteins (mg mL^{-1})	Total activity (U mL^{-1})	Specific activity (U mg^{-1})	Purification (fold)	Yield% (total activity)
Culture filtrate	200	4.18	54.18	12.97	0	100
$(NH_4)_2SO_4$ precipitation (60%)	30	2.05	48.24	23.53	1.82	89.04
DEAE-anion exchange chromatography	10	0.558	40.46	72.51	5.59	74.68
Gel permeation chromatography	4	0.089	14.36	161.35	12.44	26.50

using the GPC column as mentioned above with 50 mM Tris–HCl, pH 7.5 with 100 mM KCl; as equilibration buffer at a flow rate of 1 mL min^{-1}. 1.0 ml samples were collected for analysis. The elution volume (V_e) of the standard protein markers (Cytochrome C (12.4 kDa); Carbonic anhydrase (29 kDa); Bovine serum albumin (66 kDa); Alcohol dehydrogenase (150 kDa); β amylase (200 kDa); Blue dextran (2000 kDa)) obtained from Sigma Aldrich, USA; and the void volume (V_o) was determined. A semilog plot projecting the standard curve of molecular mass vs. V_e/V_o for each respective protein standard was plotted (Gel filtration Marker Kit, Sigma Aldrich, USA).

Chitin zymography (activity staining) was performed using a method developed by Gohel et al. (2005) with some modifications. NATIVE–PAGE with a 12% separating gel containing 0.1% acid swollen chitin obtained by reprecipitation of colloidal chitin after treating it with 25% H_2SO_4 was prepared. After loading the purified chitinase samples, electrophoresis was carried out. Further, the gel was incubated for 2 h at 50 °C in 0.1 M citrate–phosphate buffer, pH 5.5. The gel was then stained with 0.01% Calcofluor white M2R in 0.1 M citrate–phosphate buffer, pH 5.5 for 30 min, and further destained with water. The lytic zones were photographed under the UV-transilluminator in a Chemidoc MP imaging system (Biorad, USA).

The isoelectric point (*pI*) of the chitinase was determined by using IPG strips (ReadyStrip™, Biorad, USA) and the isoelectric focusing was carried out in Protean IEF Cell (Biorad, USA). For determining the *pI*, the *pI* calibration kit from Amersham (GE Healthcare Lifesciences, UK) was used. Gel permeation chromatography purified chitinase fraction was used for *pI* determination.

2.5. Effects of pH, temperature, salinity, and addition of metal ions, solvents and denaturing agents on enzyme activity

The effect of *pH* was determined by incubating the purified chitinase over a *pH* range of 1.0–11.0. The buffer systems used includes, HCl–KCl (100 mM, *pH* 1.0–2.0); citrate–phosphate (100 mM, *pH* 3.0–8.0), Tris–HCl (100 mM, *pH* 8.0–9.0) and carbonate–bicarbonate (100 mM, *pH* 9.0–11.0). The *pH* stability was tested by incubating the purified chitinase in buffers with different *pH* from 1.0 to *pH* 11.0 at standard assay temperature for 2 h. The residual enzyme activity was tested at optimum *pH*. Optimum temperature for the purified chitinase activity was determined over a temperature range of 20–80 °C. Temperature stability was tested by the pre-incubation of the enzyme at temperatures ranging from 20 °C to 80 °C for 3 h, and further assay was carried out at optimum temperature. The enzyme activity at various levels of salinity was measured over a range of 0.25–5.0% NaCl concentration in the assay mixture under conditions of optimal *pH* and temperature. The effects of metal ions on enzyme activity were assayed after the addition of metal ions Ag^{2+}, Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Hg^{2+}, K^{+}, Mg^{2+}, Mn^{2+}, Na^{+} and Zn^{2+} in the form of either their chloride or phosphate salts to the reaction mixtures. EDTA, which is a metal ion chelator was added as a negative control. The metal ions and EDTA were added to the reaction mixture so as to achieve different final concentrations of 5, 10 and 20 mM. The effects of addition of various solvents on enzyme activity was studied by the addition of acetic acid, acetone, butanol, chloroform, DMSO, ethanol, hexane, isoamyl alcohol, isopropanol, methanol and toluene at final concentrations of 10 and 20% in the assay mixture. The effects of various protein denaturants on the chitinase activity were studied. Urea was added at final concentration of 200 mM, reducing agents like β-mercaptoethanol, dithiothreitol were added at final concentrations of 10 mM and surfactants including Sodium dodecyl sulphate (SDS), Tween (20,40, 60, 80) and Triton X 100 were added at final concentrations of 1% to the assay mixture. The residual chitinase activity was measured. The chitinase activity was expressed as the percent relative activity. Appropriate controls were maintained wherever required during these studies.

2.6. Substrate specificity and enzyme kinetics

The purified chitinase was incubated with various substrates including colloidal chitin, powder chitin, powder chitosan, glycol chitosan, powder cellulose, carboxymethyl cellulose (CMC) and soluble starch. These were added to the assay mixture individually at a concentration of 1% (w/v) under standard assay conditions and the degree of substrate hydrolysis was analysed wherein the quantity of the reducing sugar released was determined by DNS method as mentioned earlier. The effect of substrate concentration on chitinase activity was studied using colloidal chitin as substrate. Colloidal chitin with a final concentration ranging from 2 to 50 mg mL^{-1} was used, and the enzyme activity was then determined by the standard assay protocol. The kinetic constants K_m and V_{max} were determined from the Lineweaver–Burk plot.

2.7. Antifungal assay of purified chitinase

Antifungal activity was assayed by the agar diffusion method using various fungal species including *Colletotrichum gleosporoides*, *Penicillium expansum*, *Pythium alphanidermatum*, *Fusarium Oxysporum* and *Penicillium chyrosogenum*. Yeast lytic activity of chitinase was assayed using *Candida albicans*. Potato dextrose agar (PDA) plates were spread plated with 0.1 mL fungal spore and yeast cell suspension having a concentration of 10^7 spores/cells per mL. Further wells of 10 mm diameter were bored into the agar. 200 μL of the purified chitinase (ion exchange chromatography purified fraction with a specific activity of 72.51 U mg^{-1}) was added to the experimental well while heat inactivated purified chitinase was added to the control well. The plates were then incubated at 37 °C for up to 6 days and observed for zones of clearance around the wells that indicated the cell wall lysis and thus growth inhibition.

3. Results and discussion

3.1. Enzyme purification

The crude enzyme obtained after fermentation process using the *Streptomyces* sp. was purified using ammonium sulphate

Table 2
Comparison of specific activity, yield and fold of purification achieved after the last purification step.

Organism	Specific activity (U mg^{-1})	Yield (%)	Purification fold	References
Streptomyces sp.	161.35	26.50	12.44	Present study
Bacillus sp.	62.4	15	5.6	Dai et al. (2011)
Bacillus licheniformis	506.55	17.15	9.84	Kudan and Pichyangkura (2009)
Brevibacillus formosus	94.21	37.7	6.1	Meena et al. (2014)
Micrococcus sp.	93.02	19.95	10.33	Annamalai et al. (2010)
Pseudomonas sp.	0.175	9	25	Wang et al. (2010)
Streptomyces sp.	2.95	1.09	6.15	Han et al. (2009)
Streptomyces sp.	506.55	17.15	9.84	Margino et al. (2010)
Streptomyces halstedii	527	37	182	Joo (2005)
Streptomyces roseolus	30	34	23	Jiang et al. (2012)

precipitation, DEAE based ion exchange chromatography and gel permeation chromatography consecutively (Table 1). Many chitinases produced by different bacterial cultures have been purified using ammonium sulphate precipitation method followed by multiple column chromatographic steps (Han et al., 2009; Wang et al., 2010; Dai et al., 2011; Rabeeth et al., 2011; Brzezinska et al., 2013). The chitinase purification carried out has been summarised in Table 1. The chitinase was 12.44-fold purified with a specific activity of 161.35 U mg^{-1} and a recovery yield of 26.5%. A comparison of specific activity, yield and fold of purification achieved after the last purification step of the chitinase from the *Streptomyces* sp. in the present study with that of other bacterial cultures has been portrayed in Table 2.

3.2. Gel electrophoresis

The molecular mass of the *Streptomyces* sp. was estimated approximately at 40 kDa and was seen on gels as a single band purified after carrying out gel permeation chromatography. Also the gel permeation chromatography based molecular weight determination method using protein standards confirmed this observation. The molecular weight calculated by this method was 39.72 kDa. In accordance with the activity staining the chitinase enzyme in the ammonium sulphate purified fraction showed a clear band by zymography, confirming that it is a chitinase. Molecular masses of bacterial chitinases is generally range from 30 to 80 kDa (Annamalai et al., 2010; Margino et al., 2010; Babashpour et al., 2012; Jiang et al., 2012; Brzezinska et al., 2013; Dai et al., 2011). The purified enzyme was analysed by SDS–PAGE and activity staining of the gel (chitin zymography) shown in Fig. 1. The molecular weight of the chitinase reported in this study is similar to that reported for two *Streptomyces* strains (Jiang et al., 2012; Pradeep et al., 2014).

The *pI* of the *Streptomyces* sp. chitinase was determined to be 6.9. According to earlier reports bacterial chitinases show a broad range of *pI* [illegible] (Hobel et al., 2005; Yong et al., 2005; Adrangi et al., 2010; Kudan and Pichyangkura, 2009; Prakash et al., 20[illegible]).

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3.3. Effect of pH and temperature on the activity and stability of the purified chitinase

pH and temperature play an important role in determining the enzyme activity and stability. The chitinase from the *Streptomyces* sp. was active from *pH* 2.0 to 7.0, exhibiting maximum activity at *pH* 2.0 and *pH* 6.0 as observed in Fig. 2. Beyond *pH* 7.0 there was a rapid decrease in the activity indicating that the enzyme is active within the acidic range. Similarly, the stability is also exhibited in the acidic *pH* range as seen in Fig. 2. The enzyme, when incubated at basic *pH*, may be denaturing irreversibly and hence a reduction in its activity was observed. Most of the purified bacterial chitinases show moderate to high activity as well as stability within the *pH* range of 4–10 (Kudan and Pichyangkura, 2009; Adrangi et al., 2010; Margino et al., 2010; Rabeeth et al., 2011; Babashpour et al., 2012; Meena et al., 2014). But the chitinase in

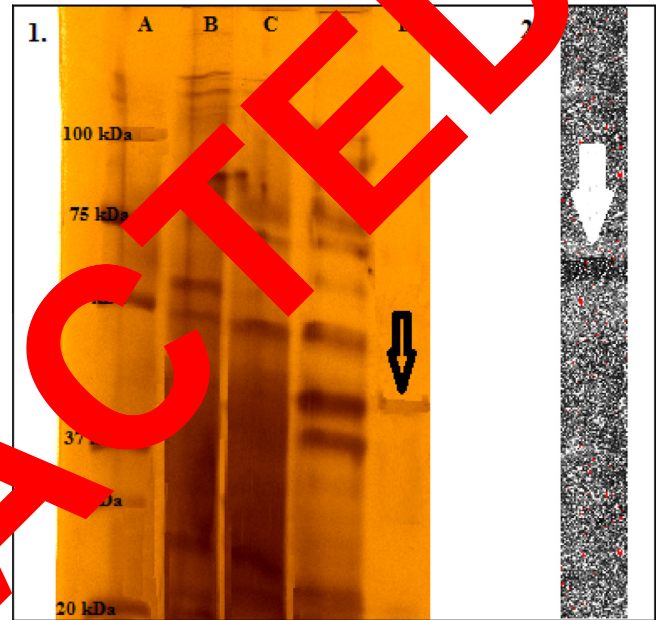

Fig. 1. SDS–PAGE analysis of chitinase enzyme. Lane A: protein standard molecular weight marker, Lane B: crude enzyme, Lane C: ammonium sulphate precipitation, Lane D: ion exchange chromatography, Lane E: gel permeation chromatography. Arrow indicates the single 40 kDa band of the purified enzyme. Chitin zymography/activity staining of chitinase enzyme.

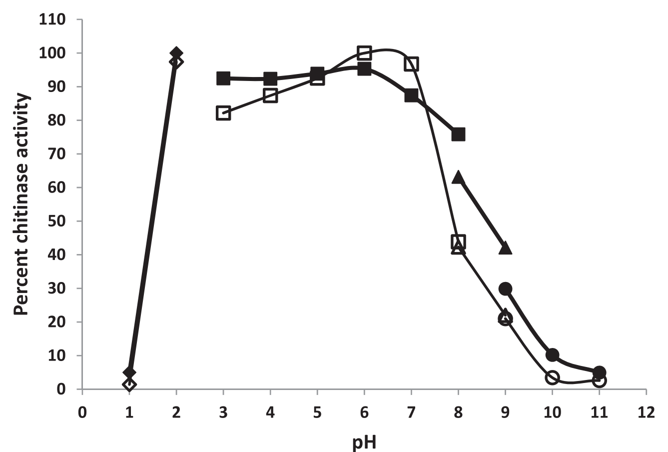

Fig. 2. *pH* optimum and stability of chitinase enzyme. Hollow shapes: *pH* optimum; filled shapes: *pH* stability; ◇ HCl–KCl buffer; □ citrate–phosphate buffer; Δ Tris–HCl buffer; ○ carbonate–bicarbonate buffer.

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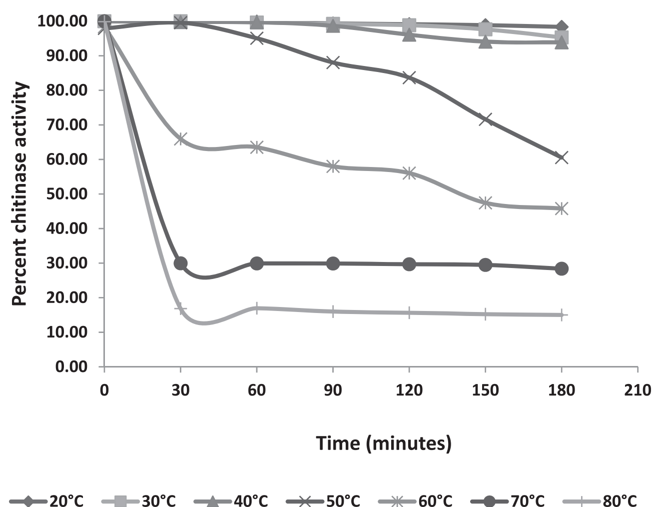

Fig. 3. Temperature stability of chitinase enzyme.

this study was found to be comparable to the acidic chitinase reported for a *Microbispora* sp. showing highest activity at *pH* 3 (Nawani et al., 2002). Also it is more stable in acidic media than the chitinase from *Rhodothermus marinus* (Hobel et al., 2005), *Sanguibacter* sp. (Yong et al., 2005) and *Streptomyces roseolus* (Jiang et al., 2012). A *Streptomyces* sp. chitinase was reported to be active in the alkaline range from *pH* 8–14 with optimal *pH* of 12.5 (Pradeep et al., 2014).

Chitinase produced by the *Streptomyces* sp. exhibited a temperature optimum at 50 °C. At temperatures below and above 50 °C, the enzyme activity reduced progressively and significantly. The activity dropped to below 50% of the highest activity at 30 and 60 °C. The chitinase exhibited high stability up to 40 °C even after three hours of incubation (Fig. 3). The residual enzyme activity reduced to around 60% after 3 h when incubated at 50 °C showing the reduced stability of the enzyme at 50 °C. At temperatures above 50 °C, the enzyme stability was significantly reduced as seen in Fig. 3. The $t_{1/2}$ value for 50 °C was calculated to be 234 min while the same for 60 °C was calculated to be 148 min. The optimum temperature for most bacterial chitinases is within 40–60 °C and show moderate activity within the range of 30–70 °C while the thermal stability is generally observed up to 50 °C (Rabeeth et al., 2011; Brzezinska et al., 2013; Kudan and Pichyangkura, 2009; Pradeep et al., 2014; Meena et al., 2014). The temperature optimal and stability of the chitinase in the present study was found similar to the chitinase from a *Streptomyces* sp. (Han et al., 2009) and a *Pseudomonas* sp. (Wang et al., 2010). Chitinases from *Bacillus licheniformis* (Toharisman et al., 2005), *R. marinus* (Hobel et al., 2005) and *Streptomyces* sp. (Prakash et al., 2013) exhibit high optimum temperature of [illegible]0 °C. Many industrial processes are operated at extremes of *pH* (either acidic or alkaline) and at elevated temperatures that make the enzyme necessary to suit the process requirements. It must also be capable of withstanding such harsh conditions for prolonged periods of the processing time. Other advantages of carrying out conversions at increased temperatures (50 °C and above) significantly reduces microbial contamination of the substrate being processed. The extreme acidic nature combined with a reasonably high optimum temperature, and its stability makes the *Streptomyces* sp. chitinase a lucrative enzyme to be used for industrial processes.

3.4. Effect of metal ion addition on the activity of the purified chitinase

Metal ions play a significant role in biological catalysis by forming complexes with the enzymes and maintaining or disrupting the 3-dimensional structure and configuration (Andreini et al., 2008). The influence of metal ions on the activities of chitinase obtained from the *Streptomyces* sp. was studied as shown in Table 3. The activity was enhanced by 15% in presence of Ca^{2+} and Fe^{2+}. It was inhibited moderately to strongly by Fe^{3+}, Cu^{2+}, Co^{2+}, Hg^{2+}, Ag^{2+} and Mn^{2+}. The activity was least affected by K^{+}, Na^{+}, $Zn^{2+}$and Mg^{2+}. Also, there was no significant inhibition of activity in the presence of EDTA. As observed for the *Streptomyces* sp. in this study, Ca^{2+} was observed to generally enhance chitinase activity as reported by Annamalai et al. (2010), Natsir et al. (2010), and Dai et al. (2011). Cu^{2+} ions are known to catalyse the auto-oxidation of cysteines to form intra-molecular disulphide bridges or the formation of sulphenic acid. Cu^{2+} was found to be a moderate to strong inhibitor of chitinases produced by different bacterial genera including *Bacillus* (Natsir et al., 2010; Waghmare and Ghosh, 2010), *Streptomyces* (Pradeep et al., 2014; Jiang et al., 2012), *Micrococcus* (Annamalai et al., 2010), *Pseudomonas* (Wang et al., 2010) and *Stenotrophomonas* (Jankiewicz et al., 2012). The major inhibitor of chitinase activity was Hg^{2+} since it reacts with –SH groups found in cysteine residues in the protein chain and disrupts the tertiary structure. Hg^{2+} strongly inhibits chitinases from different genera including *Bacillus* (Waghmare and Ghosh, 2010), *Massilia timonae* (Adrangi et al., 2010), *Microbispora* (Nawani et al., 2002), *Rhodothermus* (Hobel et al., 2005), *Stenotrophomonas* (Jankiewicz et al., 2012) and *Streptomyces* (Nagpure and Gupta, 2013; Prakash et al., 2013). The other major inhibitory metal ions which show strong inhibitory action on chitinases include Fe^{2+} (Pradeep et al., 2014; Wang et al., 2010), Mn^{2+} (Pradeep et al., 2014; Wang et al., 2010), Ag^{2+} (Nagpure and Gupta, 2013; Liu et al., 2010), Zn^{2+} (Jankiewicz et al., 2012; Prakash et al., 2013) and [illegible]$^{2+}$ (Jiang et al., 2012; Yong et al., 2005). As opposed to this Mn^{2+} moderately enhanced the activity of chitinase obtained from a *Sanguibacter* sp. (Yong et al., 2005) and a *Streptomyces* sp. (Han et al., 2009), while Zn^{2+} slightly enhanced the chitinase activity of *Bacillus* sp. (Dai et al., 2011) and a *Streptomyces* sp. (Han et al., 2009). EDTA, which is a chelating agent capable of binding metal ions in solution has been reported to reduce the activity of chitinases produced by *Bacillus* sp. (Dai et al., 2011), *Micrococcus* sp. (Annamalai et al., 2010), *Pseudomonas* sp. (Wang et al., 2010), *Streptomyces* sp. (Pradeep et al., 2014) and *M. timonae* (Adrangi et al., 2010). But it had no significant effect on chitinases produced by a *Microbispora* sp. (Nawani et al., 2002), *Bacillus thuringiensis* (Liu et al., 2010), *Streptomyces* sp. (Han et al., 2009; Nagpure and Gupta, 2013; Jiang et al., 2012).

Table 3
Effect of metal ion addition on chitinase activity.

Metal ion	Percent relative activity		
	5 mM	10 mM	20 mM
Control	100 ± 1.3		
Ag^{2+}	35.45 ± 1.6	33.12 ± 1.4	30.85 ± 0.9
Co^{2+}	71.45 ± 0.9	68.17 ± 1.3	64.87 ± 1.6
Ca^{2+}	116.32 ± 2.1	118.63 ± 1.2	107.15 ± 0.6
Cu^{2+}	100.60 ± 0.5	79.99 ± 1.3	45.34 ± 1.5
Fe^{2+}	115.38 ± 1.8	119.90 ± 1.7	122.46 ± 2.2
Fe^{3+}	91.36 ± 1.3	80.30 ± 1.9	73.97 ± 1.9
Hg^{2+}	10.35 ± 1.3	8.51 ± 0.7	7.65 ± 1.1
K^{+}	111.18 ± 1.2	97.82 ± 0.8	96.73 ± 1.3
Mg^{2+}	94.83 ± 1.5	83.24 ± 1.5	74.97 ± 1.2
Mn^{2+}	51.79 ± 1.4	50.24 ± 1.4	49.49 ± 1.6
Na^{+}	102.75 ± 1.9	101.07 ± 1.8	93.05 ± 0.7
Zn^{2+}	108.85 ± 1.6	101.98 ± 1.6	85.74 ± 1.8
EDTA	96.94 ± 1.9	94.59 ± 1.3	81.53 ± 1.4

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Table 4
Effect of solvent addition on chitinase enzyme activity.

Solvent	Percent relative activity	
	10%	20%
Control	100 ± 1.5	
Acetic acid	16.66 ± 1.9	16.98 ± 0.7
Acetone	74.11 ± 1.4	66.06 ± 1.7
Butanol	51.53 ± 1.4	45.54 ± 0.4
Chloroform	70.03 ± 1.8	74.92 ± 1.7
DMSO	58.79 ± 1.5	47.97 ± 1.9
Ethanol	55.36 ± 0.9	49.69 ± 1.1
Hexane	107.22 ± 1.7	119.91 ± 1.3
Isoamyl alcohol	71.44 ± 1.0	63.42 ± 1.3
Iso propanol	56.91 ± 1.8	49.46 ± 1.5
Methanol	57.19 ± 1.3	50.58 ± 1.6
Toluene	96.98 ± 0.7	110.62 ± 0.2

3.5. Effect of salinity on the activity of the purified chitinase

Marine micro-organisms usually are tolerant to higher salinity levels. Salt-tolerant enzymes may play significant roles in the industrial processes that require high-salt environments. Since the *Streptomyces* sp. was isolated from coastal environment samples (Karthik et al., 2013), it was expected that the chitinase expressed would be salt tolerant. The activity of the chitinase remained constant up to 1% NaCl concentration. After that, it gradually reduced to give 50% residual activity at 5% NaCl concentration. A *Streptomyces* sp. produced chitinase that showed highest activity up to 45 g% psu salinity (Han et al., 2009).

3.6. Effects of solvent addition on the activity of the purified chitinase

The effect of solvent addition on the activity of chitinase obtained from the *Streptomyces* sp. is summarized in Table 4. It was found that acetic acid strongly inhibited the chitinase activity reducing it to 17% of the original activity. Most of the solvents including butanol, chloroform, DMSO, ethanol, [illegible] isoamyl alcohol, isopropanol and methanol moderately reduced the chitinase activity. But in the presence of hexane and toluene there was a small increase in enzyme activity. It indicates that hydrophobic interactions are essential for enzyme activity. A *B. licheniformis* chitinase was reported unstable toward organic solvents such as butanol, 2-propanol, ethanol, and DMSO at a concentration of 5%, (Toharisman et al., 2005).

3.7. Effect of denaturing agent addition on the activity of the purified chitinase

The influences of denaturing agents on the activity of chitinase obtained from the *Streptomyces* sp. were studied. In the case of surfactants, chitinase activity was insignificantly affected by Tween (20, 40, 60, 80) and Triton X 100 but was affected by SDS that caused a 27% reduction in its original activity. Urea caused a decrease in activity to 86% of the original activity. β-mercaptoethanol and dithiothreitol significantly reduced the chitinase activity to 23% and 20% of the original activity respectively. Urea inhibited the activity of chitinases produced by two *Streptomyces* sp. (Han et al., 2009; Prakash et al., 2013). The activity of a *Streptomyces* sp. chitinase was moderately reduced by the presence of β-mercaptoethanol (Pradeep et al., 2014) but was enhanced as reported by Rabeeth et al. (2011) for another *Streptomyces* sp. Dithiothreitol also inhibited chitinases from *Streptomyces* sp. (Prakash et al., 2013) and *Bacillus* sp. (Dai et al., 2011). There was no effect of SDS on the activity of chitinases produced by a *B. thuringiensis* (Liu et al., 2010), *B. licheniformis* (Toharisman et al., 2005) and *Streptomyces griseus* (Rabeeth et al., 2011), while the presence of SDS increased the activity of the chitinase produced by a *Pseudomonas* sp. (Wang et al., 201[illegible]) but SDS strongly inhibited the chitinase a *Streptomyces* sp. as reported by Han et al. (2009). Tween 20 and Triton X-100 did not have any effect on the activity of *B. licheniformis* (Toharisman et al., 2005).

Table 5
Substrate specificity of chitinase enzyme.

Substrate	Specific activity (U mg^{-1})	Relative percent activity
Colloidal chitin	73.97 ± 0.9	100
Powder chitin	27.96 ± 0.7	37.8
Powder chitosan	14.59 ± 0.8	19.7
Glycol chitosan	14.79 ± 0.2	20.0
Powder cellulose	0	0
Carboxymethyl cellulose	0	0
Starch	0	0

3.8. Substrate specificity and kinetic parameters

The purified *Streptomyces* chitinase was assayed using different substrates and it showed highest activity towards colloidal chitin. A comparatively lower activity was observed in the presence of powder chitin, while it showed very feeble activity towards chitosan. It showed no activity towards cellulose, carboxymethyl cellulose and starch (Table 5). Similar was observed in case of *S. roseolus* (Jiang et al., 2012) and a *Bacillus* sp. (Dai et al., 2011) where the chitinase did not show any activity towards chitosan, cellulose, carboxymethyl cellulose and starch. A chitinase produced by *B. licheniformis* was highly active for substrates such as colloidal chitin, glycol chitin, chitosan and colloidal chitosan (Toharisman et al., 2005).

The kinetic constants K_m and V_{max} of the *Streptomyces* sp. chitinase was determined using a Lineweaver–Burk Plot to be 6.74 mg mL^{-1} and 61.3 U mg^{-1} using different concentrations of colloidal chitin (2–50 mg mL^{-1}). The K_m values were similar to that of a *Sanguibacter* sp. whose K_m was reported to be 6.95 mg mL^{-1} for colloidal chitin (Yong et al., 2005). Many of the reported K_m values for colloidal chitin were lower than that for the *Streptomyces* sp. (Joo, 2005; Han et al., 2009; Kudan and Pichyangkura, 2009; Rabeeth et al., 2011; Nagpure and Gupta, 2013), suggesting that the affinity of the enzyme for the substrate obtained in this study was different from that of chitinase from other microorganisms. V_{max} values of 6.6 U mg^{-1}, 7.03 U mg^{-1} and 180 U mL^{-1} using colloidal chitin as substrate were reported for different *Streptomyces* sp. by Nagpure and Gupta (2013), Kudan and Pichyangkura (2009) and Rabeeth et al. (2011) respectively. Yong et al. (2005) reported a V_{max} value of 10.53 U min^{-1} mg^{-1} for a *Sanguibacter* sp. while Babashpour et al. (2012) reported a V_{max} value of 10.53 U for *Serratia marcescens* using colloidal chitin as substrate.

3.9. Antifungal activity

The antifungal activity of the *Streptomyces* sp. chitinase was determined by agar plate diffusion method. Inhibition was observed on PDA plates inoculated with various phytopathogenic fungi and wells loaded with purified chitinase. The chitinase inhibits the growth of *C. gleosporoides*, *P. expansum*, *P. alphanidermatum*, *F. Oxysporum* and *P. chyrosogenum*. The hyphal inhibition was observed as a zone of clearance around the well containing the active purified chitinase while abundant growth was observed around the control well. But there was no growth inhibition observed in the case of *C. albicans* as abundant growth was seen around both the control well as well as the well containing the

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active purified chitinase. Hence it may be said that the chitinase is active only against filamentous fungi and not against yeasts. The endochitinase from *S. halstedii*, *B. thuringiensis*, *S. marcescens*, *S. griseus*, *S. maltophilia* and *S. violaceusniger* have been shown to inhibit the growth of *Fusarium graminearum*, *Bipolaris* sp., *Aspergillus brassicicola*, *Rhizoctonia solani*. *Alternaria alternata*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Phytophthora capsici*, *Colletotrichum gloeosporioides*, *Stemphylium lycopersici*, *Botrytis cinerea*, *Penicillium chrysogenum*, *Penicillium glaucum*, *Phanerochaete chrysosporium*, *Schizophyllum commune*, *Gloeophyllum trabeum*, *Coriolus versicolor* and *Pityriasis versicolor* (Joo, 2005; Liu et al., 2010; Rabeeth et al., 2011; Babashpour et al., 2012; Jankiewicz et al., 2012; Nagpure and Gupta, 2013). A *Streptomyces* sp. chitinase was reported to inhibit *C. albicans* (Han et al., 2009). The present study indicates that the chitinase from the reported *Streptomyces* sp. can be used as a promising biocontrol agent against plant pathogens.

4. Conclusions

A 40 kDa extracellular chitinase produced by the *Streptomyces* sp. was purified. The enzyme was significantly active and stable in the acidic range of pH and could tolerate significantly high salt concentrations. Temperature stability results suggest that it can be stored at room temperature for prolonged periods without significant activity loss. The chitinase has significant specificity towards colloidal chitin. The chitinase was able to inhibit the growth of phytopathogenic fungi, making it a promising candidate as a safe biopesticide. Overall findings conclude that, the chitinase from the *Streptomyces* sp. can be usefully exploited for agric[illegible] biological and environmental applications.

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Appendix A. Supplementary data

Supplementary data associated with t[illegible]rticle can be found, in the online version, at http[illegible]x.doi.org/10.1[illegible]biortech.2015.03.006.

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