

Purification and characterization of chitinase from *Streptomyces violascens* NRRL B2700

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Chitinase is one of the important enzymes as it is directly linked to Chitin that has wide applications in industrial, medical and commercial fields for its biocompatibility and biodegradability. Here, we report extracellular chitinase production by *Streptomyces violascens* NRRL B2700 under submerged fermentation condition. Chitinase production started after 10 h of incubation and reached to maximum level at 72 h of cultivation. Studies on the influence of additional carbon and nitrogen sources on chitinase production revealed that maltose, xylose, fructose, lactose, soybean meal and ammonium nitrate served as good carbon and nitrogen sources to enhance chitinase yield by 1.6 to 6 fold. Medium supplemented with 1% colloidal chitin produced high chitinase concentration (0.1714 U/mg). The enzyme chitinase was purified from the culture broth by 75% ammonium sulphate precipitation, DEAE-cellulose ion-exchange and sephadex G-100 gel filtration. The molecular mass of the purified chitinase was 65 kDa as estimated by SDS-PAGE. The apparent Michaelis constant (K_m) and the maximum rate (V_{max}) of the enzyme for colloidal chitin were 1.556 mg/mL and 2.680 μ M/min/mg, respectively suggested high affinity towards chitin. Possibly, it is the first report on production of chitinase from *S. violascens* NRRL B2700. The findings were encouraging, especially for cost effective production, and further warrants media and purification optimization studies for enhanced yield.

Keywords: Actinobacteria, Characterization, Colloidal chitin, Microbes

Chitin ($C_8H_{13}O_5N$) is a main component of fungal cell wall, exoskeleton of arthropods, nematodes, diatoms and algae components¹. As the second most abundant insoluble biodegradable polymer, it is composed of repeating units of N-acetyl D-glucosamine (N-[2,4,5-trihydroxy-6-(hydroxymethyl) tetrahydro-2H-pyran-3-yl]acetamide) residues². Chitin and its hydrolytic products have wide applications as industrial, medical and agricultural³⁻⁶. They protect plants against microorganisms by providing support for binding of protective molecules such as lectins⁷. Chitin is hydrolyzed by three hydrolytic enzymes exochitinase (chitobiosidases and β -N-acetyl hexosaminidase), endochitinase and chitobiase⁸.

Chitinase (EC number 3.2.1.14) catalyzes depolymerization of chitin by random hydrolysis of N-acetyl- β -D-glucosamine by 1-4- β -linkages and gains attention due to their biological, medical and agricultural applications⁹⁻¹³. Chitinase is a monomeric protein with the molecular weight of 21-70 kDa that

forms a single strand of polypeptide. Complete biodegradation of chitin requires synergistic action of several hydrolytic enzymes. The combined action of endochitinases and exochitinases leads to degradation of chitin polymer into the soluble N-acetyl D-glucosamine¹⁴.

Chitinases are reported from wide range of organisms, including bacteria (*Bacillus*, *Klebsiella*, *Vibrio*, *Pseudomonas*, *Serratia*), *Streptomyces*, fungi (*Aspergillus*, *Mucor*, *Trichoderma*), plants (*Arabidopsis thaliana*, *Hevea brasiliensis*, *Musa paradisica*), invertebrates and vertebrates^{9,15-17}. *Streptomyces* spp. have been shown to be the source of biologically important enzymes including chitinases^{10-12,18}. Microbes demonstrate higher chitinase productivity than animals and plants¹⁶. As chitinolytic enzymes play important role in the growth and development of insects, nematodes and fungi, chitinases could prove to be safer pesticides or biocontrol agents (mosquito control) than the chemical ones^{10,19}. Mishra *et al.*²⁰ not only provides valuable insights into binding sites of chitinase proteins and fungal resistance but also reveals the

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functional similarity of wheat chitinases with that of barley and rice. Chitinases are used for the production of chito-oligosacchrides and N-acetyl glucosamine as sweetener, plant defender, growth factor, single cell protein and anti-inflammatory drug^{14,15}.

The chemical degradation methods of chitinous waste materials incur problems of high cost and chemical waste disposal, and hence, the biodegradation of chitin is gaining importance^{15,21}. Keeping aforesaid facts in view about the significance of chitinases in various applications, we explored new strains for better productivity. Here, we present the production of chitinase from *Streptomyces violascens* strain. It is the first report demonstrating chitinase production from *S. violascens*.

Material and Methods

Micro-organisms and screening of chitin degrading strain

A pure culture of *S. violascens* acquired from Agricultural Research Service (ARS) culture collection (NRRL) was maintained on yeast malt glucose (YMG) agar medium (g/L yeast extract: 4, glucose: 4, malt extract: 10 and agar: 20). The primary screening was performed by streaking the culture on colloidal chitin agar (CCA) plate²², (chitin 0.45%, agar 1.5%) and incubating at 30°C for 5-7 days. The chitinase production was further confirmed by cup-plate assay method using 100 µl of 72 h old fermented broth on colloidal chitin (0.45%) agar^{22,23} and indicator plate²⁴.

Fermentation

Seed culture was prepared by inoculating the 50 mL X- medium (g/l: MgSO₄·7H₂O 0.5, (NH₄)₂HPO₄ 0.5, NaCl 3, K₂HPO₄ 1, soybean meal 10, CaCO₃ 3 and glycerol 15 mL) with a loop full slant culture and incubating at 28°C and 180 rpm for 48 h. About 2% (v/v) inoculum was used to inoculate the production medium of similar composition as seed medium and incubated at 28°C and 180 rpm for 96 h. Samples were collected at a regular interval of 24 h and used for enzyme assay by dinitrosalicylic acid (DNS) method²⁵. Chitin, N-acetyl D glucosamine, chitosan, cellulose and cellobiose used during different experiments of the study were purchased from Sigma Chemical Co. (St. Louis, USA).

Preparation of colloidal chitin

Colloidal chitin was prepared according to the method as described by Priya *et al.*²², with minor modifications. About 60 mL of concentrated HCl (32%)

was added slowly to 5 g of shrimp shell chitin powder and left at room temperature (28°C) for 2-3 h with vigorous stirring. Ice cold tap water was added slowly to the above mixture for precipitation and incubated overnight at 28°C further with vigorous stirring. The precipitate was collected by centrifugation at 4°C, washed with the sterile distilled water until its pH became neutral (pH 7.0). The colloidal chitin was weighed, autoclaved for 15 min at 15 psi, and stored in a dark place at 4°C.

Chitinase assay

Chitinase activity was determined by dinitrosalicylic acid (DNS) method as described by Miller²⁵, using colloidal chitin as a substrate. The culture filtrate (1 mL) was incubated with 1 mL of 0.45% colloidal chitin in a phosphate buffer (0.1 M pH 7.0) at 37°C for 30 min in shaking water bath. The reaction was stopped by adding 2 mL DNS reagent and kept in a boiling water bath for 5 min to develop the colour. The tubes were cooled, centrifuged at 5000 g for 10 min and absorbance was measured at OD 575 nm against the blank prepared with 0.1 M phosphate buffer and 0.45% colloidal chitin without enzyme. One unit of chitinase is defined as the amount of enzyme, which releases 1 µmol of N-acetylglucosamine per hour under the reaction condition²⁶. The extracellular protein concentration was measured according to Lowry *et al.*²⁷ using bovine serum albumin (BSA) as a standard²⁸.

Effect of carbon and nitrogen supplements on chitinase production

Basal production medium (X medium) was supplemented with different carbon (1% w/v) and nitrogen (organic 1%, inorganic 0.1% w/v) sources to assess the effect of C/N sources on chitinase production. All the experiments were performed in triplicate. Chitin is a complex polysaccharide, which is difficult to be utilized by microorganisms. Therefore, colloidal form of chitin was used as substrate. Different (0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 1.6%) concentrations of colloidal chitin were added to the production medium to examine its effect on chitinase production.

Purification

The purification of chitinase was carried out in three steps at 4°C as detailed below:-

Ammonium sulphate precipitation— Seventy two hours old culture filtrate was precipitated with ammonium sulphate of 75% saturation at 4°C. The precipitated proteins were collected by centrifugation

at 10000 g for 20 min. The protein pellet was dissolved in 0.01 M phosphate buffer (pH 7) and dialyzed for overnight at 4°C against the same buffer using the 12 kDa MW cut-off membrane (Sigma, USA). The dialysate was collected and used for further purification.

Anion exchange chromatography— The dialyzed enzyme was loaded on a pre-equilibrated (0.01 M phosphate buffer) DEAE-cellulose (Sigma, USA) column (1.3×16 cm). The unbound proteins were washed with two times of the bed volume (50 mL) of 0.1 M phosphate buffer (pH 7) and the enzyme was eluted with step-wise gradient of NaCl (0.1-1.1 M) in 0.01 M potassium phosphate buffer at a flow rate of 0.5 mL/min. Fractions of 3 mL were collected and tested for chitinolytic activity using DNS method. Chitinolytic fractions were pooled, concentrated and further purified by gel filtration.

Gel filtration chromatography— The concentrated fraction was loaded on a Sephadex G-100 (1.8×50 cm) column pre-equilibrated with a 0.01 M phosphate buffer (pH 7). The column was washed with the same buffer for the removal of unbound protein and eluted isocratically with NaCl (0.2 M in 0.01 M phosphate buffer) at a flow rate of 0.5 mL/min. The collected fractions were tested for chitinolytic activity. The active fractions were recovered, concentrated, dialyzed and the concentrated enzyme was kept at -20°C.

Analysis of purified enzyme by HPLC

To confirm the purity of enzyme, analysis was performed using HPLC system (Waters instrument) equipped with 515 HPLC binary pump, 2998 Photodiode array (PDA) detector and 2707 Autosampler. HPLC separation was achieved on a reverse phase, Symmetry ODS (5 µm; 250×4.6 mm) column with a mobile phase consisting of water-acetonitrile gradient at flow rate of 1 mL/min and effluent was measured at OD 280 nm.

Molecular weight determination

The active fractions were checked for purity and molecular weight determination by SDS-PAGE using Mini Protean II apparatus (Bio-Rad Laboratories Inc., USA) on 12% polyacrylamide gel following the method of Laemmli (1970)²⁹, with the standard marker (Bio-Rad Laboratories Inc., USA). Samples were compared with a standard protein marker after 90 min of separation at 120 V. Finally, the gels were stained with coomassie brilliant blue (CBB) 250.

Enzyme characterization

The purified chitinase was characterized with respect to its optimal temperature, pH, metal ions effect, surfactants and substrate specificity for enzymatic activity.

The optimum temperature for chitinase activity was determined by performing enzyme assay at different temperatures ranging from 20-70°C at pH 7 in phosphate buffer (0.1 M) using 0.45% colloidal chitin as a substrate. Chitinase stability was identified by pre-incubation of purified chitinase without substrate in 0.1 M phosphate buffer pH 7 (optimum pH), at various temperatures (20-70°C) for 1 h. Samples were collected at an interval of 10 min (up to 60 min) and relative chitinase activity was determined.

The optimum pH for chitinase activity was measured at different pH values (3-10) in 0.1 M phosphate buffer by DNS method²⁵, using colloidal chitin as a substrate. To determine the pH stability, purified chitinase was pre-incubated without substrate at 4°C with the pH range of 3-10 in 0.1 M phosphate buffer for 10-12 h and activity was measured under standard assay condition.

The effect of metal ions and surfactants on enzyme activity was observed by pre-incubating the enzyme with 0.01 M concentration of Cu²⁺ (CuSO₄), Ca²⁺ (CaCl₂), Ag⁺ (AgNO₃), Mg²⁺ (MgCl₂), Fe³⁺ (FeCl₃), Co²⁺ (CoCl₂), SDS, EDTA and Mercaptoethanol at 37°C for 1 h. The remaining chitinase activity was measured using the standard assay²⁵. The relative enzyme activity of the treated enzyme was compared with the untreated enzyme and shown in terms of percentage ratio.

Substrate specificity was investigated by incubating purified chitinase with various substrates separately, such as amylose, cellobiose, chitopentose, colloidal chitin, chitin flakes, chitosan, cyclodextrin raffinose and starch at concentration of 0.45% w/v in 0.1 M phosphate buffer (pH 7) using standard assay method²⁵. The relative activity was calculated using colloidal chitin as a control.

Michaelis and Rate Constant Determination

The effect of colloidal chitin on the chitinase activity was evaluated by ranging the colloidal chitin concentration from 0.1-1.0 mg/mL (w/v). The K_m and V_{max} values were determined by Lineweaver-Burk's plot which depicts the reciprocal of velocity (1/v) against the reciprocal of substrate concentration (1/[S]).

$$\frac{1}{V} = \left(\frac{K_m}{V_{max}} \right) \frac{1}{[S]} + \frac{1}{V_{max}}$$

The Michaelis constant or K_m , represents the substrate concentration, which results in half-maximal velocity.

Statistical analysis

All data were represented as mean ($\pm$ SD, $n=3$) and one way ANOVA was performed followed by Dunnett's test. The statistical significance level of the results was set at P value <0.05 .

Identification of the degradation product of chitin

About 1 mL of dialyzed enzyme was incubated with 1 mL of 0.45% colloidal chitin in phosphate buffer (0.1 M pH 7.0), at 37°C for 30 min in shaking water bath. The reaction was terminated by adding 1 mL acetonitrile. The tubes were centrifuged at 12000 rpm for 10 min and the supernatant was analysed for N-acetylglucosamine (NAG) formation with HPLC technique. Efficient separation was achieved on symmetry ODS (5 μ m; 100 $\times$ 4.6 mm) column with mobile phase (water: acetonitrile 31:69) at a flow rate of 1 mL/min. Effluent was detected at OD 240 nm.

Results

Screening and fermentation profile

Production of chitinase was confirmed by the presence of clear zone on chitin agar plate due to chitin hydrolysis (18 mm) and presence of purple colour zone (15 mm) on chitinase detection media (Fig. not shown). Results of time course study for microbial growth and chitinase production have been summarized in Fig. 1. Visible microbial growth was observed after 8 h, which progressively reached to maximum (6.28 g/L) around 80 h of incubation. Maximum chitinase activity 0.1523 U/ml was observed at 72 h incubation. Crude protein content was measured (935 mg/L) with specific activity 0.104 U/mg (Table 1).

Effect of carbon and nitrogen supplements on chitinase production

Table 2 presents the effects of various carbon and nitrogen sources on chitinase production by *S. violascens*. Addition of sugars, such as maltose, xylose, fructose, lactose and arabinose enhanced the production of chitinase by 5.5, 4.9, 4.29, 3.43 and 1.8 folds, respectively. Soybean meal and ammonium nitrate enhanced the production of chitinase by

1.6 folds independently. Also, supplementation of serine significantly increased the production of chitinase by 3 folds. With the increase in the concentration of inducer (colloidal chitin) from 0.2 to 1.0% (w/v) chitinase production increased regularly and maximum chitinase activity (0.14 U/mL) was observed at 1.0 % level. However, further increase in concentration of inducer decreased the chitinase activity.

Purification of chitinase

Extracellular chitinase produced from *S. violascens* was purified in two steps of chromatography, i.e., anion exchange followed by gel filtration chromatography. Anion exchange chromatogram exhibited 6 peaks (data not shown). Fifth peak eluted with 0.3 M NaCl containing chitinolytic activity was further purified by Sephadex G-100 column chromatography (Fig. 2). Quantitative results of the purification steps have been summarized in Table 1. The results demonstrated 13.7 folds purification with overall yield of 33.67 %. Purity of the enzyme was examined by SDS-PAGE and the estimated molecular

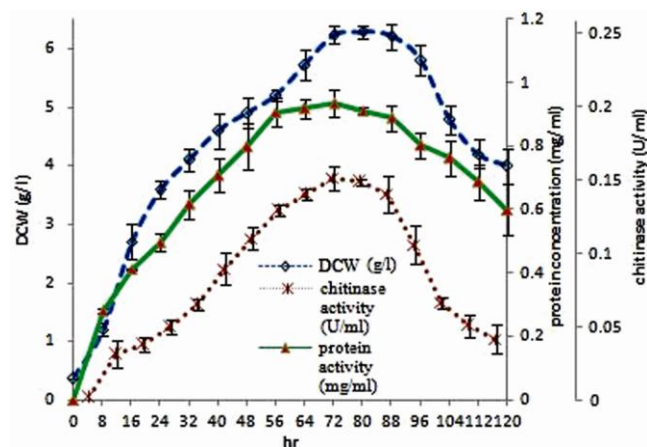


Fig. 1— Growth and fermentation profile of *S. violascens* in basal production medium. Mean values were 3 replicates. Bars represent standard error.

Table 1— Purification steps of extracellular chitinase from *Streptomyces violascens*

Purification steps	Total protein (mg)	Total activity (Unit)	Specific activity (U/mg)	Purification fold (%)	Yield (%)
Culture filtrate	935	98	0.104	1	100
Ammonium sulfate precipitation	508	84	0.165	1.58	85.71
DEAE-cellulose	103	48	0.47	4.51	48.98
Sephadex G-100	23	33	1.43	13.75	33.67

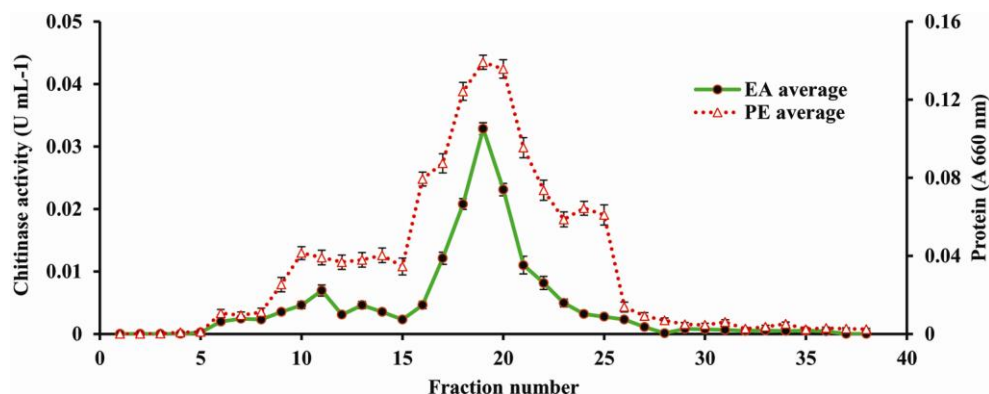


Fig. 2— Elution profile of the chitinase from Sephadex G-100 column. The protein was eluted with a 0.2 M NaCl in phosphate buffer (pH 7.0) at a flow rate of 0.3 mL/min.

Table 2— Effect of carbon and nitrogen sources as supplement to the medium

Supplements (Carbon and nitrogen)	Enzyme activity (U/ml) $\pm$ SD	Specific activity (U/ml) $\pm$ SD
Glucose	0.0877 $\pm$ 0.004	0.0516 $\pm$ 0.003
Fructose	0.3370 $\pm$ 0.021**	0.2301 $\pm$ 0.021**
Lactose	0.2694 $\pm$ 0.024**	0.2228 $\pm$ 0.024**
Arabinose	0.1462 $\pm$ 0.030**	0.1125 $\pm$ 0.031
Maltose	0.4318 $\pm$ 0.011**	0.3129 $\pm$ 0.01**
Xylose	0.3869 $\pm$ 0.023**	0.227 $\pm$ 0.02**
Beef extract	0.0145 $\pm$ 0.005**	0.0189 $\pm$ 0.005*
Malt extract	0.0607 $\pm$ 0.003	0.0640 $\pm$ 0.003
Soyabean meal	0.1283 $\pm$ 0.012*	0.1910 $\pm$ 0.05**
Peptone	0.0122 $\pm$ 0.005**	0.0169 $\pm$ 0.005**
Myco peptone	0.063 $\pm$ 0.002	0.1045 $\pm$ 0.06
Yeast extract	0.0689 $\pm$ 0.019	0.0950 $\pm$ 0.008
Chitin	0.0913 $\pm$ 0.021	0.1621 $\pm$ 0.042
Soya peptone	0.0909 $\pm$ 0.020	0.1330 $\pm$ 0.04
Ammonium nitrate	0.126 $\pm$ 0.021	0.1240 $\pm$ 0.03
Ammonium sulfate	0.067 $\pm$ 0.013	0.1290 $\pm$ 0.05
Sodium nitrate	0.061 $\pm$ 0.005	0.0720 $\pm$ 0.007
Potassium nitrate	0.078 $\pm$ 0.01	0.1150 $\pm$ 0.02
Ammonium chloride	0.060 $\pm$ 0.006	0.0751 $\pm$ 0.004
Methionine	0.0925 $\pm$ 0.012	0.1250 $\pm$ 0.02
N acetyl L cystein	0.0992 $\pm$ 0.015	0.1081 $\pm$ 0.03
Serine	0.2368 $\pm$ 0.05**	0.1990 $\pm$ 0.03**
Valine	0.06390 $\pm$ 0.01	0.0620 $\pm$ 0.002
N acetyl DL tryptophan	0.0509 $\pm$ 0.008	0.0250 $\pm$ 0.001*
Tyrosine	0.0590 $\pm$ 0.007	0.0550 $\pm$ 0.004
Control	0.0784 $\pm$ 0.005	0.0990 $\pm$ 0.007

Basal medium was supplemented with carbon (1%), nitrogen source (1 and 0.1 % for organic and inorganic nitrogen source, respectively). Data represented is mean of 3 independent experiments. * P < 0.05 and ** P < 0.001, respectively. SD= Standard deviation.

weight (MW) was found to be ~65 kDa (Fig. 3). The purity of chitinase was further verified using HPLC analytical reverse phase C-18 silica column and single peak was observed at 11.86 min retention time.

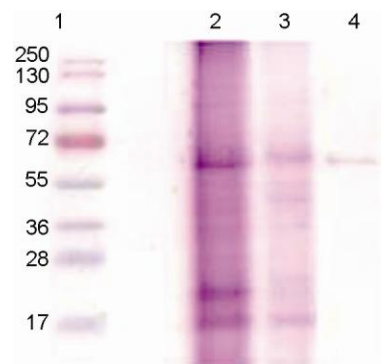


Fig. 3— SDS-PAGE of purified chitinase. [Lane 1, standard marker (250, 130, 95, 72, 55, 36, 28 and 17 kDa); Lane 2, Ammonium sulphate precipitate; Lane 3, ion exchange chromatography fraction; and Lane 4, Gel filtration chromatography fraction]

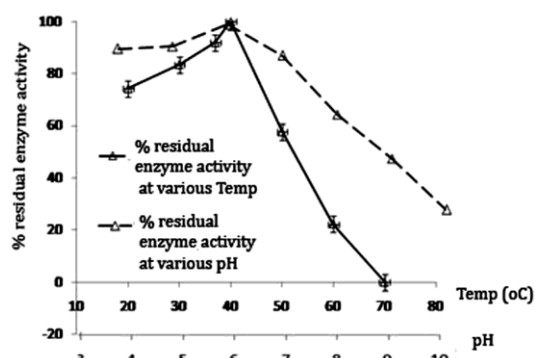


Fig. 4— Effect of temperature and pH on activity and stability of purified chitinase of *S. violascens*.

Characterization of purified chitinase

The optimum temperature for chitinase activity was 37°C. Chitinase maintained stability at pH 7 under the temperature range 20–40°C and highest stabilization was found at 40°C (Fig. 4). However, the enzyme retained more than 50% activity at 50°C and completely deactivated at 70°C. Chitinase reacted

Table 3— Effect of metals, surfactants and organic solvents on chitinase activity

Test material	Concentration (M)	Relative activity (%)
None	-	100
Copper sulphate	0.01	132.70
Calcium chloride	0.01	76.81
Silver nitrate	0.01	142.56
Magnesium chloride	0.01	79.48
Ferric chloride	0.01	80.75
Cobalt chloride	0.01	125.50
SDS	0.01	69.44
EDTA	0.01	87.79
2-ME		78.87
Chloroform		46.24
Ethanol		122.83
Methanol		85.47
Acetone		49.31

[2-ME: 2-mercaptoethanol, EDTA: ethylene di amine tetra acetic acid, SDS: sodium dodecyl sulphate]

with maximum rate at optimum temperature of 37°C. Beyond the optimum temperature, chitinase started denaturing resulting in deformation of the active site which eventually decreased the rate of reaction.

The effect of pH on chitinase activity and its stability were investigated at 37°C (Fig. 4). Chitinase exhibited optimum activity at pH 7, and it was stable between pH 3 and 10, which was higher than previous reports (pH range 3.3-7.5) of chitinase isolated from other species of *Streptomyces*³⁰.

Effect of metal ions and surfactants on chitinase activity

Cobalt (II) chloride, copper (II) sulphate and silver nitrate also enhanced chitinase activity by 25, 32 and 41%, respectively. However, in the presence of CaCl₂, MgCl₂ and FeCl₃, the enzymatic activity was reduced (Table 3). Presence of denaturing agents, such as SDS, suppressed the chitinase activity. Chelating agent (EDTA) partially inhibited the enzyme activity (relative enzyme activity 87.7%).

Substrate specificity

The hydrolysis of various substrates with chitinase showed that the purified chitinase of *S. violascens* was able to hydrolyze colloidal chitin and chitopentose exhibiting relative chitinase activity 100 and 230%, respectively. The enzyme showed little relative activity with chitosan (24%) and chitin powder (30%). However, chitinase failed to hydrolyse amylose, cellobiose, cyclodextran, raffinose and starch (0% relative activity). Double reciprocal transformation plot generates linear regressions, which allows the determination of K_m and V_{max}.

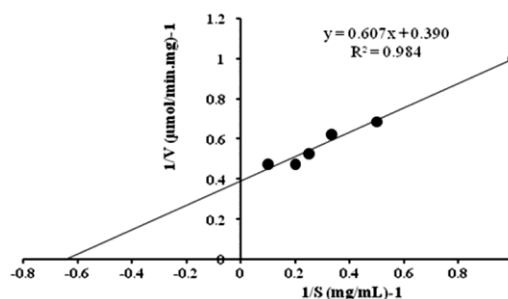


Fig. 5— Lineweaver-Burk plot of *S. violascens* chitinase reaction velocity to chitinase concentration

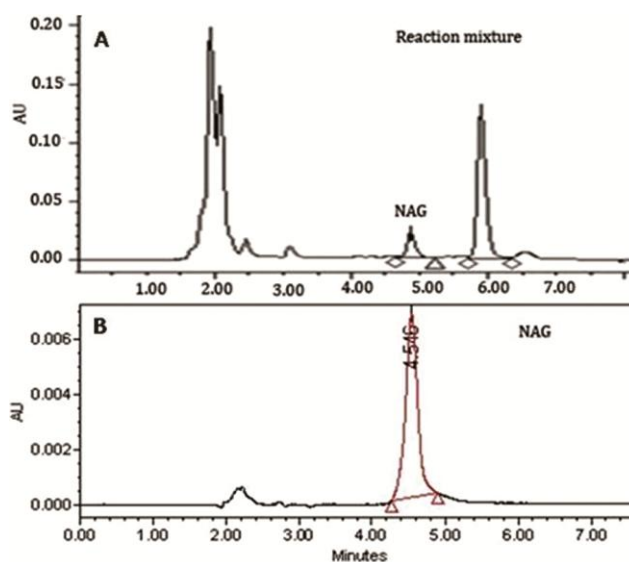


Fig. 6— HPLC chromatograms (A) chromatogram of reaction mixture showing peak of NAG at 4.54 min (B) chromatogram of pure NAG (analytical standard)

Using Lineweaver-Burk plot, V_{max} was calculated from the reciprocal of the y-intercept and it was found to be 2.680 μM/min.mg. The apparent K_m, which was calculated from the slope of the line (slope = K_m/V_{max}) was determined as 1.556 mg/mL (Fig. 5).

Identification of the chitin degradation product

The extracellular chitinase isolated from the culture broth of *S. violascens* was able to degrade chitin into N-acetylglucosamine (NAG) as a major product. HPLC analysis of the reaction mixture showed a peak of NAG at 4.546 min of retention time (Fig. 6).

Discussion

S. violascens was found to produce chitinase during the exponential phase of growth which was confirmed through clear zone on CCA plate developed due to hydrolysis of chitin and purple colour zone on

chitinase indicator (bromocresol purple) plate. Chitin hydrolysis leads to change the pH from acidic to basic, which in turn also changes the colour of medium from yellow to purple colour³¹. Among *Streptomyces* genus, *S. antibioticus*, *S. canus*, *S. griseus*, *S. pseudogriseus*, *S. plicatus*, *S. lividans* are reported as chitinase producers¹⁰⁻¹². However, chitinase production from this strain has not been reported earlier. Maximum chitinase concentration in the medium was found on the third day of incubation when the bacterium entered the stationary phase. Enzyme production gradually declined after 88 h. This decrease could be due to reduced number of viable microbial cells as result of depletion of readily available carbon and nitrogen source and other nutrients.

Chitinase was purified by ammonium sulphate protein precipitation, Anion exchange DEAE-cellulose chromatography, followed by gel filtration on Sephadex G-100 chromatography, a procedure similar to that used for *Streptomyces venezuelae*³⁰. Above process produced a purified chitinase, confirmed as a single band on SDS-PAGE with estimated molecular wt. 65 kDa which was in close agreement with the chitinases reported from *S. venezuelae* (66 kDa)³⁰. Molecular weight of microbial chitinases ranges from 20 to 120 kDa, and for *Streptomyces* it is between 30 and 68 kDa³².

In the enzyme characterization it has been observed that temperature influences the rate of enzyme-catalyzed reaction. The optimum temperature for chitinase activity was found to be 37°C. Increase in temperature from 20 to 40°C accelerated the chitinase activity which might be due to the kinetic energy supplied to the molecules leading to increased collisions between enzyme and substrate, and hence the increased reaction rate. Chitinase exhibited optimum activity at pH 7 and was stable at pH 3-10. Chitinase obtained from *Aeromonas* sp., *Arthrobacter* sp., *Alcaligenes xylosoxydans* and *Bacillus* sp. exhibited pH optima in acidic range (4.5-5.5)³²⁻³⁵. Some species of *Bacillus* and *Cellulomonas flavigena* showed optimum activity at basic pH (7, 8 and 10)^{35,36}, and yeast *Monas cuspureus* at neutral pH³⁷, similar to our present study.

The enhance chitinase activity as observed for cobalt (II) chloride, copper (II) sulphate and silver nitrate is similar to the findings of Han *et al.*³⁸ which also exhibited increased chitinase activity by Co²⁺, Cu²⁺, and Ag⁺ ions. In contrary to our results, there are several reports showing inhibition of chitinases activity by Co²⁺ and Ag⁺ ions^{28,39-41}. Suppression of

the chitinase activity by denaturing agent viz., SDS and chelating agent EDTA was similar to the observations reported by Groleau *et al.*⁴⁰ who studied peptide-peptide interactions. Isolated chitinase was able to hydrolyze colloidal chitin and chitopentaose suggesting that the enzyme possess a strong binding capability for chitin and its derivative. However, it did not hydrolyse amylose, cellobiose, cyclodextran, raffinose and starch indicating the absence of chitobiasis i.e., unable to degrade asparagine-linked glycoproteins to cleave the core chitobiose unit at its reducing end⁴².

In conclusion, chitinase was purified and characterized with an estimated molecular weight of 65 kDa. This is the first report on isolation of chitinase from *Streptomyces violascens*. Chitinase is capable of hydrolysing chitin efficiently. Broad pH tolerance and thermal stability makes the enzyme industrially significant for various biotechnological applications, especially in biodegradation of chitinous waste material and production of N-acetyl D-glucosamine that has wide use as sweetener, plant defender, growth factor, single cell protein and anti-inflammatory drug.

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