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Purification and characterization of chitinase enzyme from *Kurthia gibsonii* Mb126

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ORIGINAL RESEARCH ARTICLE

ABSTRACT

The aim of the study was to purify and characterize the chitinase enzyme from *Kurthia gibsonii* Mb126. The chitinase enzyme from *K. gibsonii* Mb126, the chitinolytic bacterial strain, was purified through four steps including ammonium sulphate precipitation, affinity adsorption, ion exchange chromatography and gel filtration chromatography. The chitinase was purified 16.11-fold through Sepadex G 100 gel filtration. The specific activity of the purified enzyme was 10.31 U/mg proteins after purification. The purified enzyme on analysis with Coomassie Brilliant Blue R 250 gave a single band near 40 KDa indicating the homogeneity of the preparation. The enzyme was most active at pH 6.5. The optimum temperature for the chitinase was 40 °C. Impacts of various metal ions, chemicals and detergents were studied. The pH stability and thermo stability of the chitinase were also studied. The chitinase exhibited K_m and V_{max} values of 11.1mg/mL and 11.12 µmoles/µg h, respectively. To the best of our knowledge, it is the first report on characterization of chitinase from *Kurthia*.

KEYWORDS
characterization; chitinase; *Kurthia*; purification

1. INTRODUCTION

Chitin is a highly insoluble β-(1, 4)-linked polymer composed of N-acetyl- glucosamine (GlcNAc). Chitinases (EC 3.2.1.14) are enzymes which can hydrolyze chitin to its oligomeric, dimeric and monomeric components. It can be classified into 2 major categories: exochitinases and endochitinases (Dahiya et al., 2005). Endochitinases (EC 3.2.1.14) cleave randomly at internal sites, generating low molecular mass multimers of N-acetylglucosamine (NAG). On the other hand, exochitinases are further divided into 2 sub categories, β-(1, 4) N-acetyl hexosaminidases (EC 3.2.1.52) and chitobiosidases (EC 3.2.1.30). β- (1, 4), N-acetyl hexosaminidases cleave the oligomeric products of endochitinases and chitobiosidases to monomers of N-acetyl glucosamine.

Chitinases have broad spectrum of distribution in nature including bacteria, fungi, plants, insects, protozoa, human and yeasts. Chitinolytic microbes occur widely in nature. They are preferred source of chitinase because of their low production cost and easy availability of raw materials for their cultivation. Bacteria like *Enterobacter* sp. (Dahiya et al., 2005), *Vibrio* sp. (Park et al., 2000), *Bacillus* (Bhushan and Hoondal 1998; Thamthiankul et al., 2001; Wen et al., 2002; Woo and Park, 2003), *Pseudomonas* (Lee et al., 2000) and *Serratia plymuthica* HRO-C48 (Frankowski et al., 2001) comprise of chitin.

Nowadays, chitinases have received increased attention because of their possible applications such as isolation of fungal protoplasts (Balasubramanian et al., 2003; Prabavathy et al., 2006), mosquito control by degrading cuticle of insects which contain chitin as an essential component (Mendonsa et al., 1996),

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production of single cell protein (Vyas and Deshpande 1991) and in the preparation of oligosaccharides and N-acetyl glucosamine (Makino et al., 2006). Purification and characterization of chitinase is important because of their wide applications in agricultural and industrial fields. Purification process is the separation of interested protein from the mixture of proteins maintaining its biological function. Most commonly used methods for chitinase purification was ammonium sulfate precipitation, affinity chromatography, ion exchange and gel filtration chromatography. Purification of chitinase has been reported from bacteria such as *Aeromonas* (Wu et al., 2001), *Bacillus* (Bhushan and Hoondal 1998; Thamthiankul et al., 2001; Wen et al., 2002; Woo and Park 2003), *Pseudomonas* (Lee et al., 2000), *Enterobacter* sp. (Dahiya et al., 2005), *Vibrio* sp. (Park et al., 2000), *Pseudomonas aeruginosa* (Wang and Chang, 1997) and *Serratia* (Duzhak et al., 2002). In this study an appropriate purification method is designed for chitinase from *K. gibsonii* Mb126, based on molecular size, electrostatic properties, and relative solubility in salts.

2. MATERIALS AND METHODS

2.1. Chemicals and substrates

Prawn waste was collected from shellfish processing units of "Kochi, Kerala, India". The chitinous waste was then sun dried, milled into small pieces of uniform size (0.6 mm mesh). Chitin and N-acetyl D-glucosamine were obtained from Hi-Media, India. Colloidal chitin was prepared by the method mentioned by Jeuniaux (1966). All other reagents used were of analytical grade.

2.2. Microorganism

K. gibsonii Mb126, the chitinolytic bacterial strain isolated from marine environments of "Kochi, Kerala, India" was used for chitinase production.

2.3. Production and purification

K. gibsonii Mb126 was cultivated in solid substrate fermentation medium where prawn shell powder (0.6mm sized) was the carbon source. The enzyme was produced at optimum conditions. The moisture content was 75%, the pH of the moistening solution was 8 and temperature of incubation was at 40 °C. The optimum inoculum size was 4-5 x 10^9 CFU/ mL. The chitinase enzyme was purified by procedures including ammonium sulfate precipitation, affinity adsorption, ion exchange chromatography and gel filtration chromatography. All these purification steps were performed at 4 °C. After each step, chitinase activity and protein content were determined.

Samples obtained at various stages of purification were run on 10% SDS– PAGE gel according to the method of Laemmli (1970).

2.4. Chitinase and protein assay

The end product, N-acetyl Glucosamine released in the supernatant was assayed by DNSA method (Miller 1959). Absorbance was measured at 530 nm using UV spectrophotometer (Shimadzu UV1800) along with substrate and blanks. One unit of chitinase was defined as the amount of enzyme that liberated one micromole of N-acetyl Glucosamine per mL per minute under experimental condition. The protein concentration was measured according to the method of Lowry et al. (1951) with bovine serum albumin as standard. For the purified enzyme, protein concentration was measured by determining the absorbance at 280 nm.

2.5. Properties of purified enzyme

The properties of purified enzyme such as optimum pH, temperature, stability at different pH and temperature, effect of salts, effect of chemicals and kinetic properties from *K. gibsonii* Mb126 were studied. Experiments were done in triplicate.

2.5.1 Effect of pH

The effect of pH on the activity of the purified enzyme was studied by measuring chitinolysis in buffers (50 mM) at different pH under standard assay conditions. Buffers used were glycine- HCl (pH 2-3), acetate (pH 3.5-5.5), phosphate (pH 6-8) and glycine -NaOH (pH 8.5-9).

2.5.2 Effect of temperature

Chitinase activity was tested at different temperatures ranging from 20 – 80 °C in 50 mM phosphate buffer (pH 6.5) using colloidal chitin as the substrate.

2.5.3 Effect of salts

The effect of various salts on the enzyme activity was studied. The enzyme was incubated with different concentrations of various salts (2-10 mM) in phosphate buffer (pH 6.5) at 40 °C for 1 h and the enzyme activities were determined. The enzyme activity in buffer without any of these salts (control) was also determined.

2.5.4 Effect of various chemicals

The effect of various chemicals such as detergents (Triton X-100, Tween-80, Tween-20 and SDS), mercaptoethanol, EDTA and H_2O_2 were studied by adding them at different concentrations (2 - 10 mM) into the assay system. The enzyme activity in buffer without any of these chemical (control) was also determined.

2.5.5 Effect of pH on stability

The effect of pH on the stability of chitinase was also studied. The enzyme was incubated in 50 mM acetate (pH 4 - 5.5) and phosphate (pH 6-7.5) buffers for 1 h at 25 °C. Activities were determined before and after incubation.

2.5.6 Effect of temperature on stability

Thermal stability was examined by exposure of the enzyme solution in phosphate buffer (50 mM, pH 6.5) at different temperature for 2 h after which the enzyme solution was cooled rapidly. Enzyme activities were determined before and after heat treatments. The percentages of activities remaining after the heat treatments were calculated.

2.5.7 Kinetic properties

Different concentrations of substrate (colloidal chitin) was added to assay system containing 0.5 mL enzyme at its optimum pH (6.5) and temperature (40 °C) and incubated for 1 h before the velocity of enzyme was measured. The amount of monomer released was extrapolated from the standard graph of N-acetyl glucosamine. The K_m (the substrate concentration at which the reaction velocity is half maximum) and V_{max} (velocity maximum of the enzyme reaction) were determined by plotting reciprocals of reaction velocity (1/V) and substrate concentration (1/S) according to the Lineweaver-Burk's (LB) plot. The y intercept of the plot is equivalent to the inverse of maximum reaction velocity (V_{max}); the x-intercept of the graph represents $-1/K_m$. The plot provides a useful graphical method for analysis of the Michaelis-Menten equation.

$$V = V_{max}[S]/(K_m+[S]) \quad (1)$$

3. RESULTS

3.1. Purification of chitinase

When the culture filtrate was subjected to fractional ammonium sulfate precipitation, chitinase was precipitated at 75% saturation. The precipitate obtained was dissolved, dialyzed and subjected to affinity adsorption chromatography. After affinity adsorption, protein was loaded into DEAE cellulose ion exchange column and eluted with 20 mM citrate-phosphate buffer (pH 5.5) NaCl gradient. The elution profile of chitinase from DEAE cellulose column is shown in Figure 1. In the chromatogram, the chitinase activity could be seen in a single peak. The peak was eluted with 20 mM citrate-phosphate buffer (pH 5.5) containing 0.2-0.4 M NaCl. Further purification of chitinase was performed by gel filtration on Sephadex G -100 column. The elution profile of gel filtration is shown in Figure 2. In the gel filtration, two major peaks of proteins were observed but chitinase activity was observed only in the second peak. The summary of purification is given in Table 1. The ammonium sulfate precipitated and dialyzed protein was measured as 1978.2 mg with specific activity of 1.87 U/mg. The protein was purified 10 fold with 27% recovery through affinity chromatography. The semi purified enzyme was further purified through the DEAE cellulose column. Chitinase activity was detected in the 0.2 to 0.4 M NaCl step-elution fraction. The active fractions were pooled, resulting in 12.93 fold total purification with a specific activity of 8.28 U/mg and 22.46% recovery of protein. The chitinase was purified 16.11-fold by Sephadex G- 100 gel filtration. The specific activity of the purified enzyme was 10.31 U/mg proteins after purification. The purified enzyme on SDS PAGE gave a single band near 40 kDa indicating the homogeneity of the preparation.

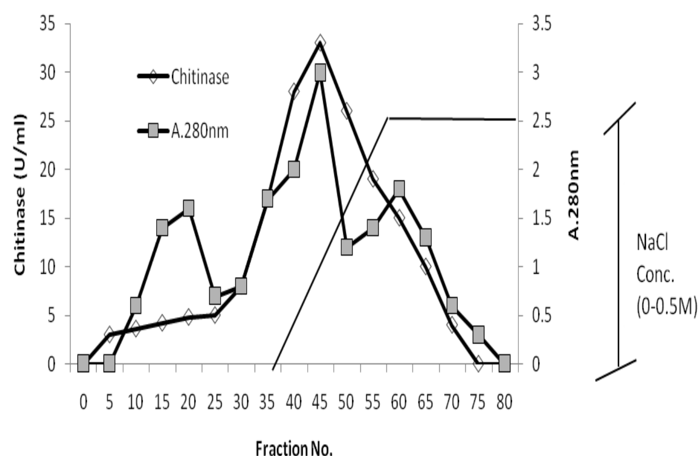

Figure 1. Chromatogram of the chitinase on DEAE cellulose ion exchange chromatography

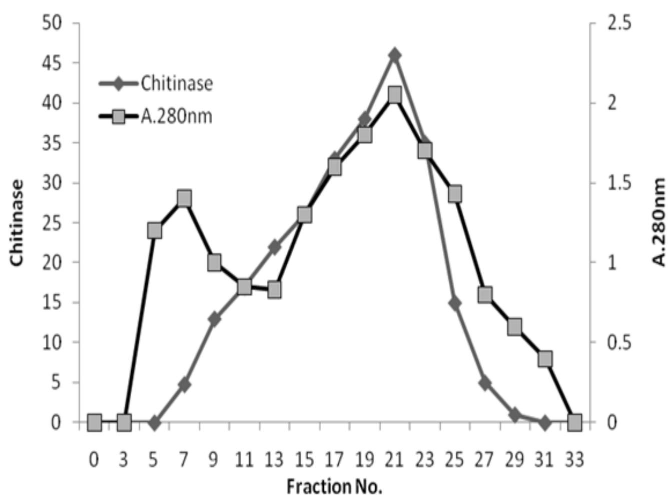

Figure 2. Chromatogram of the chitinase on a Sephadex g 100 gel fltration chromatography

3.2. Properties of purified enzyme

The enzyme was most active at pH 6.5 and the optimum temperature for the chitinase was 40 °C. The effect of pH on chitinase activity is shown in Figure 3 and the effect of temperature in Figure 4. Effect of salts on chitinase activity is shown in Table 2. Among the various salts evaluated for their effect on chitinase activity, Na^+ and Ca^{2+} enhanced chitinase activity up to 4 mM level. Further increase in concentration led to the decreased chitinase activity. The effects of Ag^+, Mg^{2+}, Mn^{2+} and Fe^{3+} on chitinase activity were not significant. On the other hand, Hg^{2+}, Cu^{2+} and Zn^{2+} inhibited enzyme significantly. There was no enzyme activity when Hg^{2+} was used at a level 6 mM and above. Results of the study on the impact of various chemicals were presented in Table 3. Among the detergents studied, Triton X-100 and SDS reduced enzyme activity significantly. Whereas, β-mercapto ethanol and dithiothreitol enhanced enzyme activity but no further increase in activity was observed with increase in concentration. H_2O_2 had inhibitory effect on chitinase activity. EDTA up to 4mM concentration did not affect the enzyme activity, above this level there was a decrease in the activity. In an effort to determine the stability of chitinase at different pH conditions revealed that the enzyme was stable between pH 4 to 7 (Figure 5). Stability profile of enzyme at various temperatures is shown in Figure 6. The purified enzyme was fairly stable for 2 h at 50 °C. Whereas, the enzyme could retain its activity completely after incubation for 40 min at 60 °C. Kinetic studies were performed for chitinase activity using colloidal chitin as substrate and the data obtained is presented in LB plot (Figure 7). The chitinase exhibited K_m and V_{max} values of 11.1 mg/mL, 11.12 µmoles/µg h, respectively.

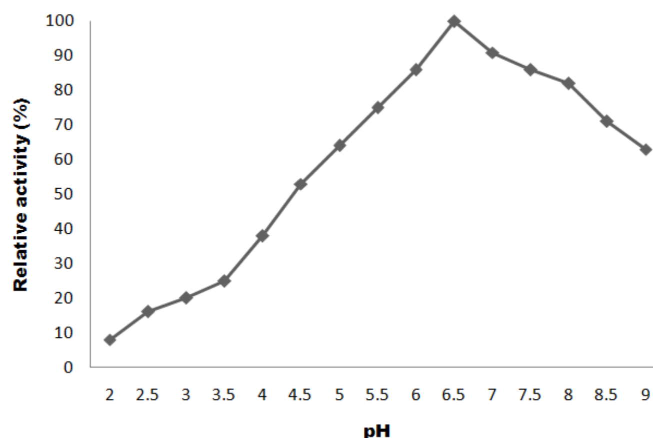

Figure 3. Effect of pH on chitinase activity

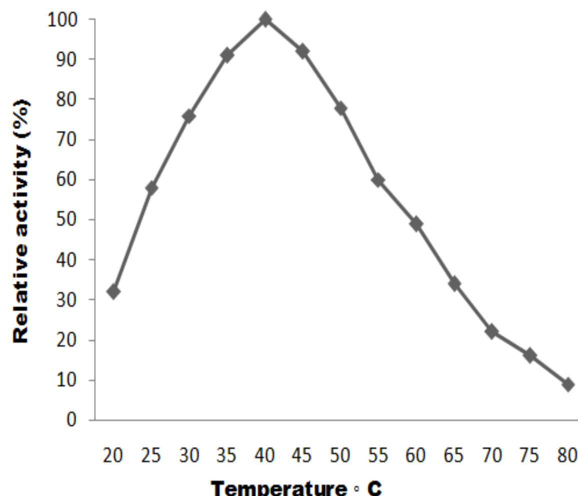

Figure 4. Effect of temperature on chitinase activity

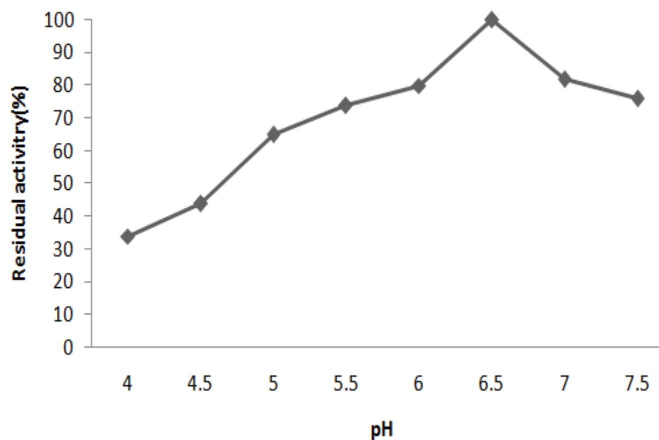

Figure 5. Stability profile of enzyme in different pH

Table 1. Summary of purification

Step	Total protein (mg)	Total chitinase activity (U/mg)	Specific activity (U/mg protein)	Purification fold	Yield (%)
Crude extract	6372.1	4078.2	0.64	1	100
$(NH_4)_2SO_4$ precipitation	1978.2	3710	1.87	2.92	90.97
Affinity adsorption	178.01	1139.6	6.4	10	27
DEAE-cellulose	110.6	916.3	8.28	12.93	22.46
Sephadex G 100	86.1	886.9	10.31	16.11	21.74

Table 2. Effect of salts on chitinase activity

Salts	Relative activity enzyme activity (%) at different concentrations*				
	2 mM	4 mM	6 mM	8 mM	10 mM
NaCl	184	199	124	63	21
KCl	69	82	93	82	45
$AgNO_2$	65	56	32	25	32
$CaCl_2$	87	95	68	45	65
$CuCl_2$	16	4	7	12	3
$HgCl_2$	8	5	0.3	0	0
$MgCl_2$	34	54	54	43	47
$MnCl_2$	34	23	24	32	15
$ZnCl_2$	12	8	7	3	2
$FeCl_2$	23	24	43	34	15

*Activity of the control is taken as 100%

Table 3. Effect of various chemicals on chitinase activity

Chemicals	Relative enzyme activity (%)* at different concentrations				
	2 mM	4 mM	6 mM	8 mM	10 mM
Triton X-100	93	45	42	32	17
Tween- 20	123	121	121	124	126
Tween- 80	46	32	43	42	45
SDS	32	45	22	16	8
EDTA	64	68	16	10	12
β- mercaptoethanol	104	146	124	130	111
Dithiothreitol	167	132	133	122	125
H_2O_2	8	6	9	6	8

*Activity of the control is taken as 100%

4. DISCUSSION

An extra cellular chitinase secreted by *K. gibsonii* Mb126 was purified to homogeneity by combination of ammonium sulfate precipitation, DEAE cellulose ion exchange chromatography, affinity adsorption column chromatography and Sephadex G-100 gel filtration chromatography. Analysis of purified chitinase showed a single band of 40 kDa size on 10% SDS-PAGE indicating the complete purification of the enzyme. Many bacteria produce only one type of chitinase; however some produce several types which differ in their size. For example, *Serratia marcescens* produced several chitinases of molecular sizes 62, 54, 43, 38 and 21 kDa (Duzhak et al., 2002), while only one chitinase was detected in the culture supernatant of *Bacillus* sp. WY22 (Woo and Park 2003). *Bacillus thuringiensis* sub sp. pakistani also gave several chitinases of 66,

60, 47 and 32 kDa in size (Thamthiankul et al., 2001). The molecular weights of chitinases from other sources were 60 kDa from *Enterobacter* sp. (Dahiya et al., 2005), 98 kDa from *Vibrio* sp. (Park et al., 2000) and 32 kDa from *Pseudomonas aeruginosa* (Wang and Chang, 1997). The enzyme showed good activity between temperatures of 30 to 45 °C, with optimal activity at 40 °C. As temperature increases, more bonds, especially the weaker hydrogen and ionic bonds, will break as a result of this strain. Breaking bonds within the enzyme will cause the active site to change shape. The rate of reaction will increase initially with increase in temperature, because of increased kinetic energy. At 40 °C, more enzymes molecules active sites shapes will be more complementary to the shape of their substrate. As temperature increases the effect of bond breaking will become greater and greater, eventually, the enzyme will become denatured and will no longer function. The temperature optima reported for other bacterial chitinases were 45 °C in *Enterobacter* sp.NRG4 (Dahiya et al., 2005) and *Vibrio* sp. (Park et al., 2000) and 55 °C in the case of *Serratia plymuthica* HRO-C48 (Frankowski et al., 2001).

The enzyme also maintained good activity in a range of pH from 6.0 to 8.0, with optimum activity at pH 6.5. At pH 6.5, the bonds within them are influenced by H^+ and OH^- ions in such a way that the shape of their active site is most complementary to the shape of their substrate. At the optimum pH, the rate of reaction is at optimum condition. Similar pH optima for other bacterial chitinases were pH 6.6 for *Serratia plymuthica* HRO-C48 (Frankowski et al., 2001), 6.3 for *Bacillus* sp. NCTU2 (Wen et al., 2002), 6 for *Enterobacter* sp. G-1 (Park et al.,1997), 5.8 for *Bacillus cereus* (Wang et al., 2001), 5 for *Aeromonas* sp. DYU-T OO7 (Lien et al., 2007) and *Ralstonia* sp. A-471 (Ueda et al., 2005) and pH 3.0 for *Microbispora* sp. V2 (Nawani et al., 2002). Regarding stability, the enzyme was retaining 100% activity at pH 6.5 when the enzyme was incubated at 40 °C and retained 100% activity at 60 °C for 40 min at pH 6.5. Chitinases from *Arthrobacter* sp. NHBN-10 (Okazaki et al., 1999) and *Vibrio alginolyticus* (Ohishi et al., 1996) are stable only at temperatures between 40 and 50 °C.

Among the various salts evaluated for their effect on chitinase activity, Na^+ and Ca^{2+} (upto 4 mM level) enhanced chitinase activity. While Hg^{2+}, Cu^{2+} and Zn^{2+} inhibited the enzyme. But in the case of chitinase of *Pseudomonas aeruginosa*, Mg^{2+} and Na^+ were inhibitory while Cu^{2+} improved the enzyme activity (Wang and Chang 1997). Reports on the effect of metal ions on chitinase are quite diverse. Generally, most chitinases were inhibited by Zn^{2+} and Cu^{2+} ions (Guo et al., 2005; Konagaya et al., 2006).

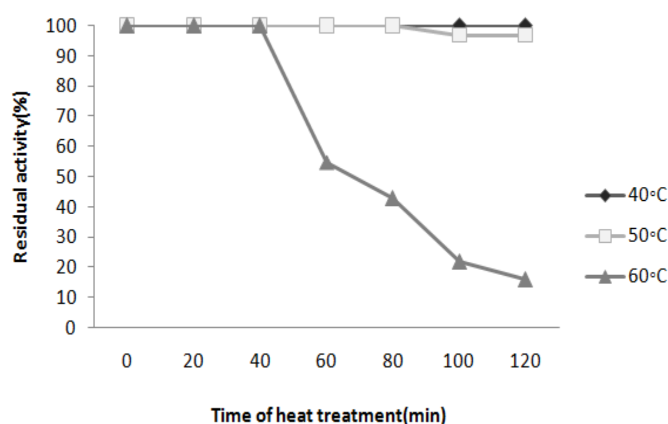

Figure 6. Stability profile of enzyme at various temperatures

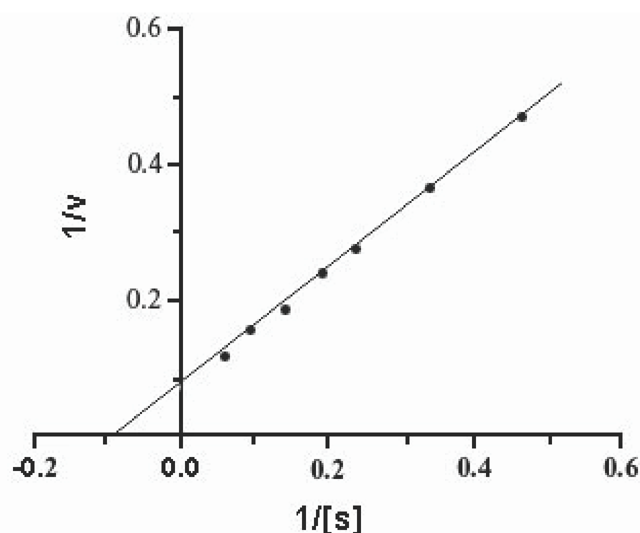

Figure 7. LB Plot showing K_m and V_{max}

There was a slight stimulation of enzyme activity in the presence of dithiothreitol (DTT) and β-mercaptoethanol. The non-inhibition of enzyme activity by dithiothreitol (DTT) and β-mercaptoethanol indicates that cysteine residue(s) do not take part in catalysis. The enzyme activation by DTT and β-mercaptoethanol can be assumed to be due to the reduction in aggregate size by destroying the intermolecular disulfide linkages and/or by the protection of thiol groups that stabilize the three dimensional structure of enzyme as suggested by Khedher et al (2008). Similar results were reported in the case of *Streptomyces viridificans* (Gupta et al., 1995) and *Streptomyces griseus* (Rabeeth et al., 2011). H_2O_2 had inhibitory effect on chitinase activity. This may be due to the oxidative damage caused by

the reactive species liberated by the decomposition. Considerable decrease in enzyme activity was also observed in the case of EDTA, which demonstrates that the purified enzyme as a type of metalloenzyme (Ramirez- Zavala et al., 2004).

5. CONCLUSIONS

Chitinase of *K. gibsonii* Mb126 had ability to hydrolyze prawn shell powder efficiently and was stable for 40 min at 60 °C. The properties such as the optimal activity at pH 6.5 and at temperature 40 °C make the enzyme suitable for environmental or biotechnological applications. Since the ambient temperatures in tropical countries are around this range, such applications will be more effective in these countries compared to the colder countries. To the best of our knowledge, it is the first report on characterization of chitinase from *Kurthia* sp.

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