J. Microbiol. Biotechnol. (2009), **19**(10), 1184–1190
doi: 10.4014/jmb.0901.029
First published online 18 August 2009

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Purification and Characterization of Endo-β-1,4 Mannanase from *Aspergillus niger gr* for Application in Food Processing Industry

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Received: January 15, 2009 / Revised: March 25, 2009 / Accepted: April 4, 2009

A thermostable extracellular β-mannanase from the culture supernatant of a fungus *Aspergillus niger gr* was purified to homogeneity. SDS–PAGE of the purified enzyme showed a single protein band of molecular mass 66 kDa. The β-mannanase exhibited optimum catalytic activity at pH 5.5 and 55°C. It was thermostable at 55°C, and retained 50% activity after 6 h at 55°C. The enzyme was stable at a pH range of 3.0 to 7.0. The metal ions Hg^{2+}, Cu^{2+}, and Ag^{2+} inhibited complete enzyme activity. The inhibitors tested, EDTA, PMSF, and 1,10-phenanthroline, did not inhibit the enzyme activity. *N*-Bromosuccinimide completely inhibited enzyme activity. The relative substrate specificity of enzyme towards the various mannans is in the order of locust bean gum>guar gum>copra mannan, with K_m of 0.11, 0.28, and 0.33 mg/ml, respectively. Since the enzyme is active over a wide range of pH and temperature, it could find potential use in the food-processing industry.

Keywords: *Aspergillus niger gr*, food processing, β-mannanase, purification

Mannan and mannan-based polysaccharides consist of a backbone of β-1,4-linked mannose residues. The complete degradation of these polymers requires an array of enzymes including endo-β-1,4-mannanases (E.C. 3.2.1.78), β-mannosidase (E.C. 3.2.1.25), and α-galactosidase (E.C. 3.2.1.22). β-Mannanase is an important enzyme for the depolymerization of these polymers. It hydrolyzes the β-1,4-linkages within the mannan backbone, releasing mannooligosaccharides of various lengths [9]. Over the years, there has been increasing interest in the potential application of β-1,4-mannanases in various industrial processes. This triggered research interest into the biochemical properties of these enzymes. As a result, β-mannanases have been purified from both bacterial and fungal sources [1, 8, 26]. The β-mannanases reported so far exhibit acidic to neutral pH optima, molecular mass ranging from 33 to 90 kDa, and mesophillic to moderately thermophilic temperature optima [2, 16, 22]. β-Mannanases are currently used in various industries such as in laundry detergents, poultry feeds, coffee processing, and in paper and pulp industries together with xylanases [18, 19, 24, 25].

Recently, we reported that the strains *Aspergillus niger gr* and *Aspergillus flavus gr* were excellent producers of extracellular β-mannanase [14]. *Aspergillus niger gr* is an GRAS organism, and the products from this strain can be used in food applications. In view of the fact that the strain was one of the best naturally occurring producers of β-mannanase, it was of interest to investigate the properties of mannanase produced by this species as a possible source of thermostable enzyme. In the present investigation, we report the purification and characterization of endo-1,4-β-mannanase from *Aspergillus niger gr*.

Materials and Methods

Microorganism and Production of Enzyme

The fungus *Aspergillus niger gr* capable of producing extracellular β-mannanase was isolated in our laboratory and used for the production of enzyme [14]. It was maintained on PDA (potato dextrose agar) slant and stored at 4°C. The previously optimized medium containing 2% locust bean gum (LBG), guar gum, and defatted copra meal were used as carbon source for mannanase production using an *A. niger gr* strain. The sterilized Erlenmeyer flasks (250 ml capacity containing 50 ml of medium) were inoculated with 7-day-old grown culture of *A. niger gr*. Batch and submerged fermentations were carried out on a rotary orbital shaker at 120 rpm and 40°C for 5 days. After 5 days of growth, mycelia were removed by filtration through Whatman filter paper No.1. The culture filtrate thus obtained was used as enzyme source and was kept at 4°C for further studies [14].

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Enzyme Assay

β-Mannanase was assayed using 0.5% (w/v) locust bean gum as a substrate. The enzyme sample (0.1 ml) was incubated with 0.9 ml of the substrate solution at 50°C for 20 min. The amount of reducing sugars produced in the enzyme reaction was measured as D-mannose reducing equivalents by the 3,5-dinitrosalicylic acid method [15].

One unit of mannanase activity was defined as the amount of enzyme that produced 1 micromole of reducing sugar as a D-mannose standard per minute under the conditions decribed above.

Protein Assay

Protein concentration was determined by the method of Lowry *et al.* [13]. Bovine serum albumin was used as a standard. The protein eluted during column chromatography was monitored by absorbance at 280 nm.

Purification of β-Mannanase

Ammonium sulfate $[(NH_4)_2SO_4]$ fractionation and dialysis. A volume of 250 ml of crude mannanase was taken initially and then the required quantity of ammonium sulfate was added slowly to obtain various saturation levels (0–40%, 40–60%, and 60–80%). The addition of ammonium sulfate was done under constant stirring at 4°C for 30 min and then stirring was continued for another 30 min. After this, the mixture was left to settle for 3 h at 4°C. Proteins were precipitated and then separated by centrifugation at 10,000 ×*g* at 4°C for 30 min. The separated proteins were then dissolved in a minimum amount of 0.05 M acetate buffer (pH 5) and refrigerated for further analysis. Precipitated proteins were transferred into a dialysis tube (Sigma) using a micropipette and dialyzed against acetate buffer (0.05 M, pH 5) at 4°C. The buffer was stirred gently using a magnetic stirrer to enhance solute exchange. Dialysis was conducted overnight and the buffer was changed several times to increase the efficiency of the dialysis.

DEAE–Sephacel chromatography. The dialyzed enzyme was loaded onto a DEAE–Sephacel column (1.9×12 cm) equilibrated with Tris-HCl buffer (0.05 M, pH 7.5) and the column was washed with the same buffer. The enzyme bound to DEAE–Sephacel was eluted with a linear salt gradient (0.5 M NaCl) in the equilibration buffer (50 mM, pH 7.5). Fractions were collected at a flow rate of 40 ml/h using a peristaltic pump (Pharmacia), and fractions (3 ml) were collected using a fraction collector. All fractions were checked for protein (A_{280}) and β-mannanase activities (A_{405}). The fractions (16^{th} to 26^{th}) having maximum enzyme activity were pooled. The enzyme was concentrated by lyophilization, dissolved in sodium acetate buffer (50 mM, pH 5.0), and dialyzed.

Zymogram analysis and molecular mass determination by SDS–PAGE. Electrophoresis under nondenaturing conditions was performed in 10% (w/v) acrylamide slab gel using a Tris-glycine buffer, pH 8.3, to examine the final enzyme preparation for its purity. Protein bands were stained with Coomassie Brilliant Blue R-250. For zymogram analysis, a substrate gel was prepared by adding 2% agarose to a 0.5% locust bean gum solution and heating until the agarose dissolved. The mixture was cast into a glass Petri-dish and allowed to solidify. After electrophoresis, the native gel was placed on the substrate gel. The two gels were then incubated at 50°C for 1 h followed by staining of the substrate gel using 0.1% (w/v) Congo red solution for 2 h. The gel was destained using 1 M NaCl and then transferred to 0.5% acetic acid to enhance the clarity of hydrolysis zones that indicated β-mannanase activity.

SDS–PAGE was conducted with the purified mannanase for elucidation of the molecular mass of enzyme protein. Electrophoresis was done on 12% gel according to Laemmli [12] and the separated protein band was detected by Coomassie blue staining. The separating gel consisted of 12% polyacrylamide and the stacking gel consisted of 5% polyacrylamide. The sample (12 μl) and 8 μl of sample buffer with 50 mM Tris HCl (pH 6.8), 10% SDS, 0.1% bromophenol blue, β-mercaptoethanol, and glycerol were loaded into the well. Molecular mass markers were purchased from Bio-Rad Laboratories, India Pvt. Ltd, and were run parallel to the samples. An electric current of 50 V was supplied using a standard power pack. The gel was stained using Coomassie brilliant blue and then destained using a mixture of methanol, glacial acetic acid, and distilled water.

Biochemical Characterization of β-Mannanase

Determination of optimum pH and pH stability. The optimum pH of enzyme activity was examined at pH 3.0–10.0 under standard assay conditions. Fifty mM of various buffers were used: acetate (pH 3.0–6.0), phosphate (pH 6.0–8.0), and glycine NaOH (pH 8.0–10.0). The enzyme reaction was incubated at 50°C for 20 min in the presence of 0.5% LBG dissolved in the buffers. The effect of pH on enzyme stability was determined using the same buffer system in the pH range of 3.0–10.0. After incubation of the enzyme solution at various pH values for 24 h at 4°C without the substrate, the remaining enzyme activity was measured at pH and temperature optima a 20 min.

Determination of optimum temperature and temperature stability. The effect of temperature on enzyme activity was performed at temperatures ranging from 30–60°C in 50 mM acetate buffer at optimum pH for 20 min. Thermal stability of the enzyme was determined at various temperatures in 50 mM buffer at optimal pH for 20 min. Then, the remaining enzyme activity was measured.

Determination of kinetic parameters, K_m and V_{max}. The Michaelis–Menten kinetic parameters K_m and V_{max} were calculated for mannanase. The K_m and V_{max} values were estimated by using LBG, guar gum, or copra mannan substrate. These reactions were performed with various concentrations of substrate from 0.1–1% (w/v) under the optimum conditions and their activities followed every 5 min for 30 min.

Determination of effects of metal ions and inhibitors. The effects of various metal ions (EDTA, Ca^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Ni^{2+}, Co^{+}, Hg^{2+}, Ag^{2+}, etc.) and inhibitors (mercaptoethanol, 1,10-phenanthroline, *N*-bromosuccinimide) on the enzyme activity were determined by measuring the activity of the enzyme in the presence of 1 mM of each ion or inhibitor in the reaction mixture using 10 mM acetate buffer, pH 5.5, under the optimum conditions.

Determination of substrate specificity. The activity of purified enzyme on 0.5% (w/v) LBG, guar gum, or copra meal was determined under the optimum conditions.

RESULTS AND DISCUSSION

Purification of β-Mannanase

The purification of mannan-degrading enzymes is based in a number of alternative combinations of procedures, such as ultrafiltration, ammonium sulfate precipitation, gel filtration, hydrophobic interaction, and ion-exchange

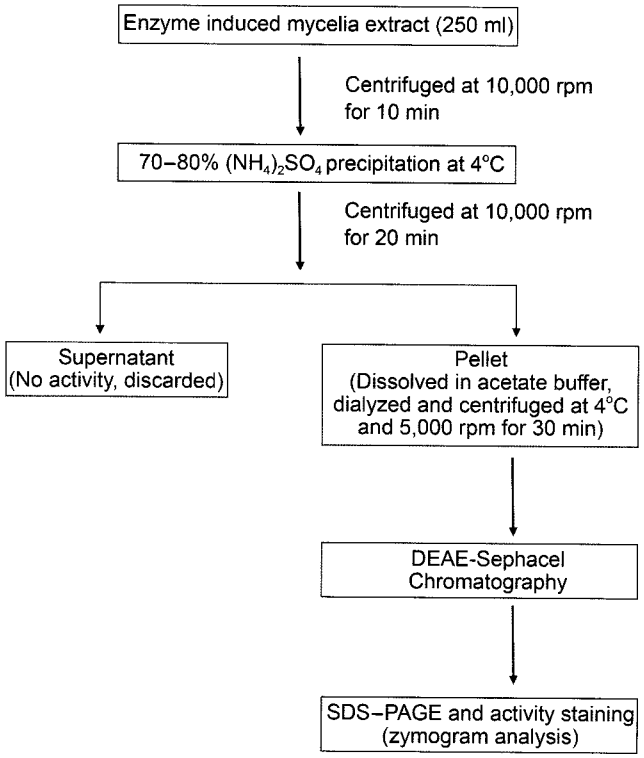

Fig. 1. Schematic representation of steps employed for the purification of endo-β-1,4-mannanase from *Aspergillus niger gr.*

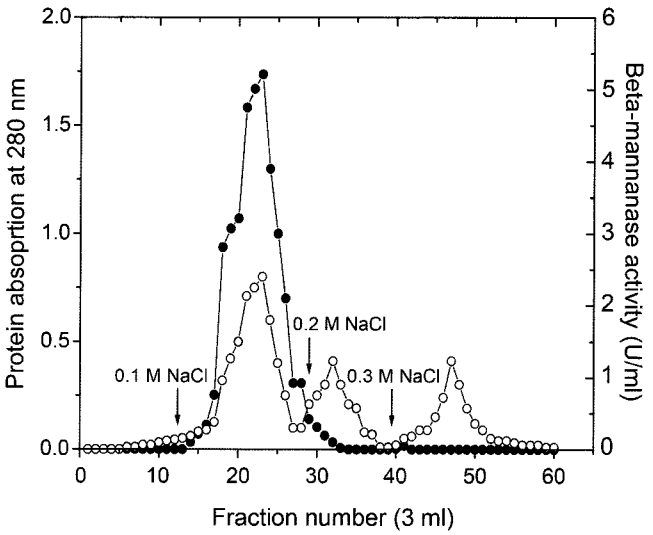

Fig. 2. Elution profile of DEAE–Sephacel column chromatography (1.9×12 cm) of β-mannanase from *A. niger gr* β-mannanase; (●—●) activity (○—○) 280 nm.

chromatography. The choice is often made empirically and confirmed by laboratory-scale experiments [3].

The complete process of purification is illustrated in Fig. 1. The fungus, cultivated in defatted copra mannan-based medium for 5 days, produces extracellular β-mannanase that was subjected to ammonium sulfate salt precipitation at 0–40%, 40–60%, 60–70%, and 70–80% saturation. After 70–80% salt precipitation, approximately 11% yield was obtained. The precipitate was dissolved in the acetate buffer (pH 5) and dialyzed against the same buffer at 4°C for 12 h. After desalting by dialysis, approximately 10% yield with 6.4-fold of purification was obtained.

The elution pattern of β-mannanase using salt gradient is shown in Fig. 2. β-Mannanase activity was observed in fraction numbers 16 to 26. The active fractions (16 to 26) were pooled and dialyzed against acetate buffer (pH 5.0, 25 mM) at 10°C for 12 h, with intermittent change of buffer. After a second step of purification (DEAE–Sephacel chromatography), 7.82-fold of purification and overall yield of approximately 10% were observed (Table 1). The dialyzate from ion-exchange chromatography was concentrated to a small volume and subjected to gel filtration on a PD-10 column. β-Mannanase secreted extracellularly by *Aspergillus niger gr* was purified to homogeneity by ammonium sulfate precipitation and ion-exchange chromatography. The procedure yielded 1.46 mg of purified enzyme from 250 ml of culture filtrate, and the recovery of total β-mannanase activity was 9.5% (Table 1).

After simple purification steps, PAGE and SDS–PAGE of the final enzyme preparation showed a single band. The molecular mass of the purified enzyme by SDS–PAGE was estimated to be between 65 and 66 kDa (Fig. 3A). Similar to our results, the molecular masses of β-mannanases purified from *Aspergillus niger*, *Aspergillus fumigatus*, *Aspergillus oryzae*, *Aspergillus tamari*, *Aspergillus aculeatus*, and *Aspergillus sulpureus* range from 48–110 kDa [5–7, 16, 17].

Biochemical Characterization of β-Mannanase

Effect of pH on β-mannanase activity and stability. From Fig. 4A, it is clear that purified β-mannanase from *Aspergillus niger gr* is optimally active at pH 5.5, and thereafter activity of the enzyme gradually decreased. From the literature, it is evident that β-mannanase from

Table 1. Summary of purification of β-mannanase.

Purification step	Total volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold purification
Crude extract	250	1,000	120.2	8.32	100	1
70–80% [$(NH_4)_2SO_4$] precipitation	10	110	2.1	52.38	11	6.3
DEAE–Sephacel chromatography	5	95	1.46	65.06	9.5	7.82

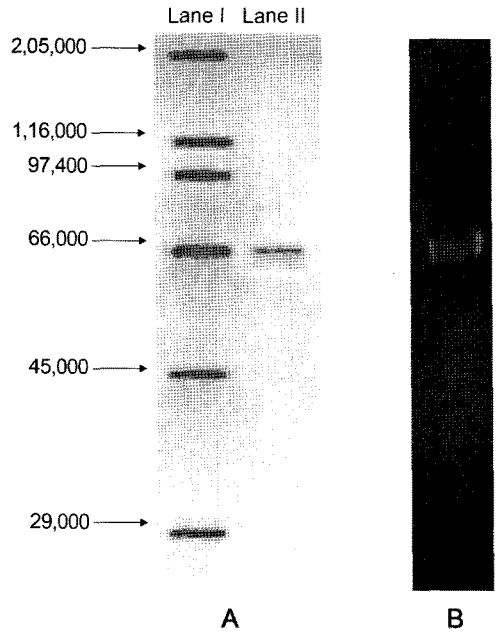

Fig. 3. SDS–PAGE of endo-β-1,4-mannanase (**A**), and activity staining (**B**).
A. Lane I: Standard mixture of myosin (205 kDa), β-Galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine albumin (66 kDa), egg Albumin (45 kDa), and carbonic anhydrase (29 kDa). Lane II: Purified β-mannanase (5 μg).

Aspergillus species has optimum activity at an acidic pH range. β-Mannanase purified from different fungal sources like *A. awamori*, *A. niger*, *A. oryzae*, *A. tamarii*, *A. aculeatus*, *A. fumigatus*, *A. sulphureus*, and *A. terrus* had an optimum pH of 3.0, 3.0, 6.0, 4.5, 5.0, 4.5, 2.4, and 7.5, respectively [5–7, 11, 16, 17, 20]. The slightly acidic pH optima of the purified β-mannanase matched the values characteristic for the fungal glycoside hydrolases [16]. Almost more than 80% of its original activity was retained after incubating the enzyme at various pH (pH range of 3–8) at 55°C up to 2 h (Fig. 4B). β-Mannanase from *S. rolfsii* was stable in the buffer at pH between 3.0 and 6.0 [10, 18]. Setati *et al* [20] have reported that β-mannanase from *S. cerevisiae* is stable between pH 4.0 and 6.0.

Effect of temperature on activity and stability of β-mannanase activity. Fig. 5A depicts temperature optima for *Aspergillus niger gr* β-mannanase. Enzyme maximally hydrolyzed LBG at 55°C (pH 5.5). β-Mannanase purified from *A. awamori*, *A. niger*, *A. oryzae*, *A. tamarii*, *A. aculeatus*, *A. fumigatus*, *A. sulphureus*, and *A. terrus* had an optimum temperature of 80, 50, 40, 60, 70, 60, 50, and 55°C, respectively [5–7, 11, 16, 17, 20]. Similarly, β-mannanase purified from the fungal strains *Trichoderma harzianum T4* and *T. reesei* exhibit optimum activity at temperature 65°C and 70°C, respectively. β-Mannanase produced by the genus *Aspergillus*, and indeed most fungi, have temperature optima within the range of 40–80°C. Fig. 5B shows the

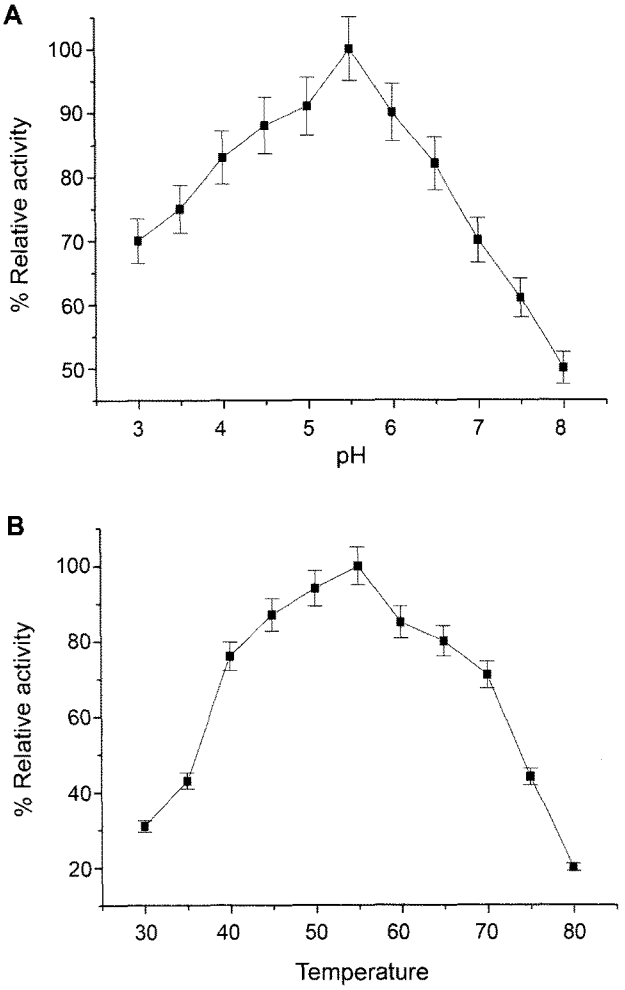

Fig. 4. Effects of pH (**A**) and temperature (**B**) on β-mannanase activity.

thermostability profile of β-mannanase. It is clear from Fig. 5B that the enzyme is 80% stable at 55°C for 4 h. Approximately 50% of activity was retained at 55°C for 6 h. The half-life of the enzyme was 6 h at 55°C and only 10% of activity was retained after 12 h incubation at 55°C. β-Mannanase from *A. aculeatus* was thermostable in the buffer at 70°C for a 2-h incubation period [6]. Similarly, β-mannanases from *A. fumigatus*, *T. reesei*, *A. sulfurous*, and *P. chrososporium* are stable at 55, 60, 40, and 60°C for a 2 h incubation period, respectively [4, 5, 16, 22].

Substrate specificity and determination of kinetic parameters. Kinetic parameters like K_m and V_{max} of *Aspergillus niger gr* β-mannanase are summarized in Table 2; the results revealed that β-mannanase had a higher affinity towards LBG (K_m, 0.11 mg/ml) than that of other mannans like guar gum and copra mannan. The relative substrate specificity of β-mannanase towards the various natural mannans was in the order of locust bean gum>guar gum>copra mannan (Table 2). Kinetic study revealed that

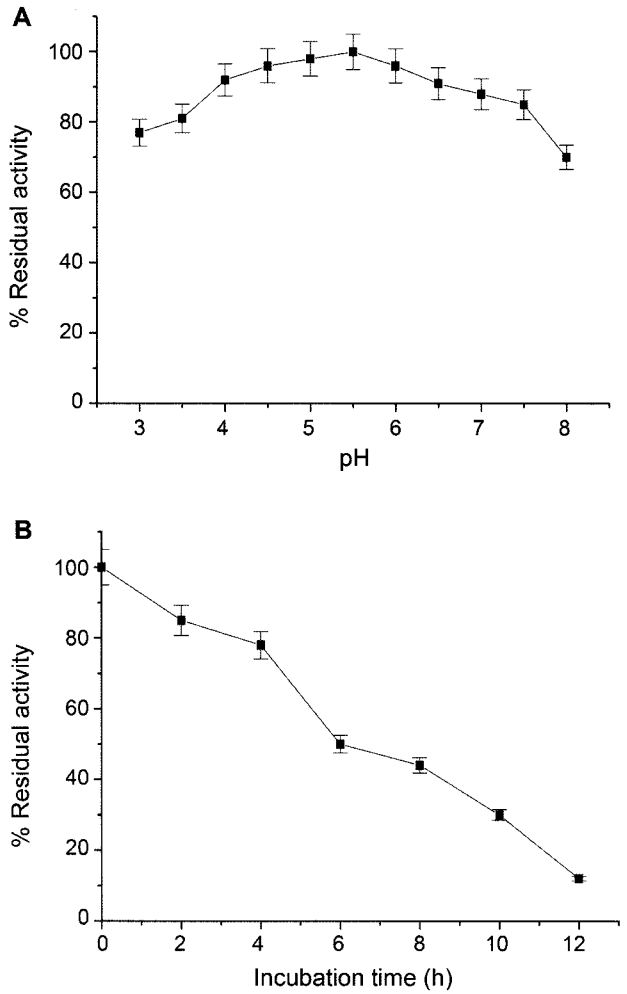

Fig. 5. Effects of pH (**A**) and temperature (**B**) on β-mannanase stability.

the enzyme prefers LBG rather than guar gum and copra mannan. Therefore, the enzyme has more affinity towards natural mannans and hence is applicable in the food-processing industry for the production of oligosaccharides. In the literature, β-mannanases from different organisms have different K_m and V_{max} values [9].

Effects of inhibitors and metal ions on β-mannanase activity. The effects of different inhibitors and metal ions on β-mannanase activity is depicted in Table 3. Among

Table 2. K_m and V_{max} values of *Aspergillus niger gr* endo-β-1,4-mannanase.

Substrates	Relative activity (%)	K_m (mg/ml)	V_{max} (μmol/ml/min)
LBG	100±5	0.11±0.00	14.13±0.70
Guar gum	90±4.5	0.28±0.01	11.23±0.56
Copra mannan	67±3.35	0.33±0.01	7.2±0.36

Values are averages±standard deviations for two independent experiments.

Table 3. Effects of inhibitors and metal ions on *Aspergillus niger gr* endo-β-1,4-mannanase activity.

Inhibitors/metal ions	Residual activity (%)
None	100±5
1,10-Phenanthroline	100±5
N-Bromosuccinimide	0±0
PMSF	100±5
EDTA	100±5
Mercaptoethanol	100±5
Urea	100±5
KCl	100±5
Mg^{2+}	100±5
Hg^{2+}	0±0
Zn^{2+}	68±3.4
Cu^{2+}	0±
Ag^{2+}	0±0
Ni^{2+}	100±5
Co^{2+}	100±5
Mn^{2+}	100±5
Ca^{2+}	120±6

Values are averages±standard deviations for two independent experiments.

inhibitors tested, EDTA and 1,10-phenanthroline did not inhibit the β-mannanase activity, and this indicates that β-mannanase is not a metalloenzyme. Inhibition of β-mannanase from *Aspergillus niger gr* by *N*-bromosuccinimide indicates the role of tryptophan at or near the active site. Inhibition of β-mannanase in *Bacillus* sp. by *N*-bromosuccinimide was also noticed [23]. The inhibition of some enzymes by NBS is caused by oxidation of Trp residues in proteins [21]. From Table 3, it is also evident that PMSF did not inhibit the β-mannanase, and this indicates the absence of a serine group at or near the active site of the enzyme. Among the metal ions tested on β-mannanase activity, mercuric (Hg^{2+}) ions completely inhibited enzyme activity. Inhibition by Hg^{2+} suggests that the enzyme contains an essential sulfydryl group. Significant inhibitory effect was also observed in the presence of Zn^{2+} and Ag^{+}. Similar to our results, β-mannanase from *Bacillus* sp. was strongly inhibited by Ag^{+} and Hg^{2+} [23]. Metals such as Mg^{2+}, Ni^{2+}, Co^{2+}, Mn^{2+}, and Ca^{2+} had no effect on enzyme action.

Table 4 depicts the biochemical properties exhibited by β-mannanases from various fungal sources in comparison with the present investigation.

β-Mannanase is an industrially important enzyme that is used mainly in the food and feed industry. As the range of applications of this enzyme is very wide, there is always a scope for novel β-mannanase with better characteristics, which may be suitable in the diverse fields of applications [17]. The present paper reports the purification of a β-mannanase from *A. niger gr.* The purified enzyme is a monomeric protein of 66 kDa and it is found to give its optimum activity at temperature 55°C. Furthermore, it was

Table 4. Thermostable β-mannanases purified from different fungi.

Organism	Optimum temperature and pH for activity		Thermostability (°C)[a]	Molecular mass (kDa)	References
	Temperature (°C)	pH			
A. niger	60	3.0	<70	56	[17]
S. rolfsii	74	2.9	<50	61.2	[10]
A. aculcatus	60–70	5.0	<70	45	[6]
A. fumigates	60	4.5	55	60, 63	[16]
T. reesei	70	3.0–4.0	60	53, 51	[22]
A. sulfurous	50	2.4	40	48	[5]
P. chrysosporium	60–70	4.0–6.0	60	65	[4]
A. niger gr	55	5.5	55	66	Present study

[a]Half-life of the enzyme was 6 h at different temperatures.

noted that the enzyme was active over a wide range of pH and temperature values. The purified enzyme showed low K_m towards natural substrates like LBG, guar gum, and copra mannan. This property of purified enzyme can be exploited in its application in food and feed industries for the production of mannooligosaccharides, and fungus *A. niger gr* does not produce toxins; their metabolic products enjoy generally recognized as safe (GRAS) status, and this can be used in the food industry.

Acknowledgment

K. Naganagouda wishes to thank the Indian Council of Medical Research (ICMR), New-Delhi, India for its financial support in the form of a Senior Research Fellowship (SRF) during this work.

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