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Partial Purification and Characterization of Thermostable Alkaline β-Mannanase from *Bacillus* sp. JB-99 Suitable for Pulp Bleaching

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Abstract *Bacillus* sp. JB-99, when grown in a chemically defined medium containing lactose as a carbon source, yielded 3,860 U/ml extracellular β-mannanase, which was high compared to other examined carbon sources. Among the nitrogen sources, yeast extract enhanced the enzyme activity. The enzyme production was growth-associated. The enzyme was optimally active at 65°C, pH 10, and had a half-life of 190 min at 65°C. N-Bromosuccinamide and $AgNO_3$, $CuSO_4$, and $HgCl_2$ strongly inhibited the enzyme, whereas Ca^{2+} stimulated the enzyme activity. The α-galactosidase enzyme production was not found in any of the enzyme assays.

Key words: *Bacillus* sp. JB 99, locust bean gum, thermoalkaline β-mannanase, pulp bleaching

β-Mannanase [1,4-β-D-mannan mannanohydrolase, (EC. 3.2.1.78)] liberates mannooligosaccharides from β-mannans and various heteropolysaccharides by hydrolyzing the β-1,4 glycosidic bonds in the backbone [14]. The enzyme is used in the total removal of galactoglucomannans present in softwood sulfite liquors [16]. β-Mannanase finds extensive applications, particularly in depolymerizing plant polysaccharides. These polysaccharides include softwood galactoglucomannans, hydrolysis of which has great industrial importance in paper and pulp bleaching. The enzyme is used for the removal of hemicellulose and thus decreases the use of toxic Cl_2 and ClO_2 in the bleaching of pulp [6]. Highly viscous seed galactomannans are used in the food industry as feed additives to enhance nutritional value by hydrolyzing the polysaccharides. [2]. The enzyme also finds applications in the preparation of mannanooligosaccharides that could be used, like other oligosaccharides, as important food additives [12]. The presence of β-mannanase in xylanase preparations that are without cellulase enhances the bleachability of pulp in paper manufacturing industries, and decreases the severe environmental damage caused by the presence of toxic and highly refractive organic byproducts in bleaching effluents [3]. In the present study, *Bacillus* sp. JB-99 was characterized for the production of thermostable alkaline β-mannanase.

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MATERIALS AND METHODS

Bacillus sp. JB-99 [9] was grown in a chemically defined medium containing (g l^{-1}) lactose 10.0, $NaNO_3$ 10.0, K_2HPO_4 5.0, $MgSO_4$ 0.3, $CaCl_2$ 0.2, and NaCl 5.0. Sodium carbonate (10 g l^{-1}) was added separately after autoclaving. Batches of submerged fermentations were carried out in 250-ml Erlenmeyer flasks containing 50 ml of the above medium, and the flasks were incubated at 50°C on a shaker at a constant speed of 200 rpm for 24 h. The culture was centrifuged at 8,000 rpm for 10 min (4°C) and the supernatant was used for further studies.

β-Mannanase activity was measured at 65°C using the 3,5-dinitrosalicylic acid (DNS) method. One ml reaction mixture contained 0.5 ml of 0.1% (w/v) Locust bean gum (LBG, Sigma, U.S.A.), 0.4 ml of 50 mM glycine-NaOH buffer (pH 10.0), and 0.1 ml of enzyme solution. The reaction was arrested after 10 min of incubation by adding 1 ml of DNS followed by boiling for 5 min. The absorbance was measured at 540 nm after diluting the colored complex with 8 ml of distilled water. One unit of enzyme activity was defined as the amount of enzyme that could release 1 μmol of D-mannose per minute under the above standard conditions [11]. The total protein concentrations of the samples were determined by the Lowry method with BSA as a standard.

An assay of α-D-galactosidase was done, following the modified method of Dey and Pridham [4]. One ml

of reaction mixture contained 0.1 ml of enzyme, with 0.4 ml of glycine-NaOH buffer (50 mM, pH 10) and 0.5 ml of p-nitrophenyl α-D-galactopyranoside (2 mM) as a substrate. The mixture was incubated for 15 min at 65°C. The reaction was terminated by adding 3 ml of 0.2 M Na_2CO_3 solution. The absorbance was measured at 405 nm. One unit of enzyme was defined as the release of 1 μmol of p-nitrophenol from p-nitrophenyl-α-D galactopyranoside (PNPG) as a substrate in standard assay conditions.

The various carbon sources were screened by replacing lactose from the above medium at different concentrations between 0.5 to 3.0% (w/v). The effect of nitrogen sources was studied by replacing sodium nitrate with organic and inorganic nitrogen sources at 0.5 to 2.0% (w/v) concentrations, where 2.0% lactose (w/v) served as the sole carbon source. The growth profile and time course of maximum enzyme production were also examined in the media containing lactose (2.0%, w/v) and yeast extract (1.0%, w/v). For all the above experiments, logarithmically grown cultures were used as inoculum. All the data presented here represent the average of three assays.

The supernatant obtained by centrifuging the 24 h grown culture for 10 min at 8,000 rpm (4°C) was used as a source of enzymes. The supernatant was saturated with 80% (w/v) ammonium sulfate and kept undisturbed for 12 h at 4°C, followed by centrifugation at 12,000 rpm for 30 min (4°C). The precipitate thus obtained was dissolved in 2.5 ml of glycine-NaOH buffer (50 mM, pH 10.0) and dialyzed against the same buffer (10 mM, pH 10.0) for 24 h at 4°C with intermittent changes of the buffer.

The optimum temperature for β-mannanase activity was determined by incubating the enzyme for 10 min at 20°C to 80°C, with 0.5 ml of 0.1% (w/v) LBG in 50 mM glycine-NaOH buffer at pH 10, and enzyme stability was studied by incubating the enzyme in the absence of substrate for 30 min and 60 min, at pH 10. The half-life of the enzyme was monitored at 65°C, pH 10 [17]. The pH profile of β-mannanase was evaluated by incubating the enzyme for 10 min at 65°C in appropriate buffers [sodium acetate buffer (pH 3.6–5.5, 50 mM); sodium phosphate buffer (pH 6.0–8.0, 50 mM); and glycine-NaOH buffer (pH 8.0–10.6, 50 mM)]. In order to determine the pH stability, the enzyme was incubated for 4 h in the absence of substrate. The residual activity was determined as stated above.

The effects of various metal ions and inhibitors on enzyme activity were examined by incubating 100 μl of enzyme solution with each of the metal ions and inhibitors at a concentration of 10^{-3} mol l^{-1} in 50 mM glycine-NaOH buffer (pH 10) at 40°C for 30 min. The residual activity was determined under standard conditions, as described above.

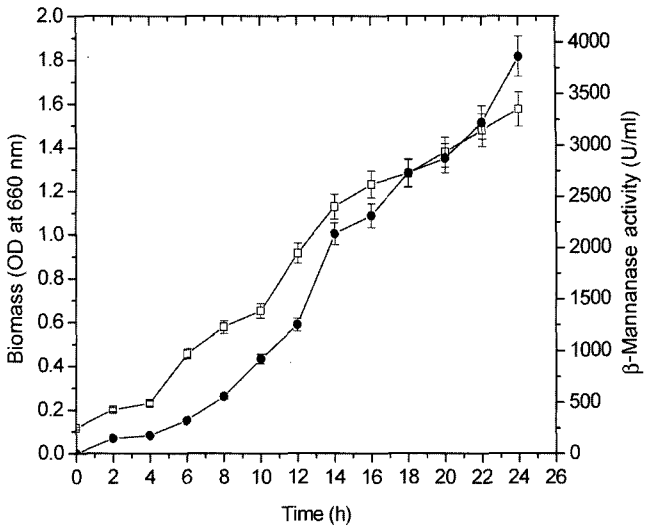

Fig. 1. Growth profile (□) and time course of enzyme (●) production.

RESULTS AND DISCUSSION

The fermentations were monitored at defined time intervals, from 2 h to 36 h. As shown in Fig. 1, it was evident that the enzyme production depended on the growth of the bacteria. Maximum production was achieved at 24 h, from where on it gradually declined (data not shown). The locust bean gum is a galactomannan containing 23% of galactose and 77% of mannose. Galactosidase cleaves galactose that is present in the side chain, and the mannooligosaccharides are cleaved into mannose by the action of β-mannanse [1]. However, in the present investigation, the α-galactosidase activity was not found in any of the culture extracts.

Table 1 shows that among the carbohydrates tested, lactose served as the best carbon source. The effect of different nitrogen sources on β-mannanase production is displayed in Table 2; among all the organic and inorganic compounds,

Table 1. Effect of various carbon sources on β-mannanase.

Carbon source (2.0%)	Enzyme activity (U/ml)
Mannose	2,164
Rhamnose	1,988
Lactose	3,860
Dextrose	616
Raffinose	322
Maltose	672
Inositol	237
Sorbitol	234
Cellobiose	848
Ribose	234
Fructose	746
Xylan	117

Each value is the mean of three assays.

Table 2. Effect of various nitrogen sources on enzyme activity.

Nitrogen source (1.0%)	Enzyme activity (U/ml)
KNO_3	66
Casein	2,807
NH_4NO_3	120
Beef extract	3,392
NH_4Cl	175
Yeast extract	4,005
Peptone	526
$(NH_4)_2SO_4$	409
$NaNO_3$	3,840
Urea	1,374
Gelatin	292

Each value is the mean of three assays.

the highest activity was achieved with yeast extract and the lowest activity was recorded with KNO_3.

The effects of temperature on enzyme activity and stability are shown in Fig. 2. The enzyme was active between 20°C to 80°C and optimally active at 65°C. The enzyme retained 90%, 80%, and 50% of its original activity after incubating for 30 min, 60 min, and 190 min, respectively, and was completely inactive at 90°C (data not shown). The above results were confirmed by repeating each experiment three times. β-Mannanase from hyperthermophilic eubacterium *Thermotoga neapolitana* [5] was one of the thermostable enzymes highly active at 100°C to 103°C. A highly thermostable endo-(1,4)-beta-mannanase from the marine bacterium *Rhodothermus marinus* ATCC 43812 had an optimum condition of 85°C at pH 5.4 [15]. β-Mannanase from *Aspergillus awamori* K4 having the optimum temperature of 80°C at pH 5 was reported by Kurakake and Komaki [11]. The

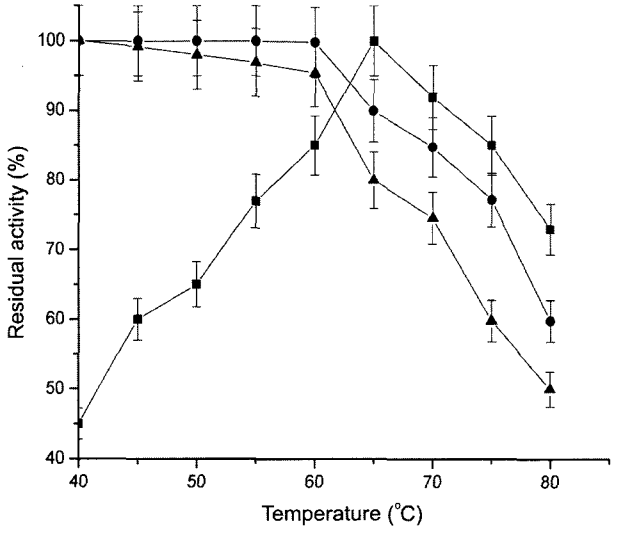

Fig. 2. Effect of temperature on enzyme activity (■); thermostability of the enzyme after 30 min (●) and 60 min (▲) of incubation.

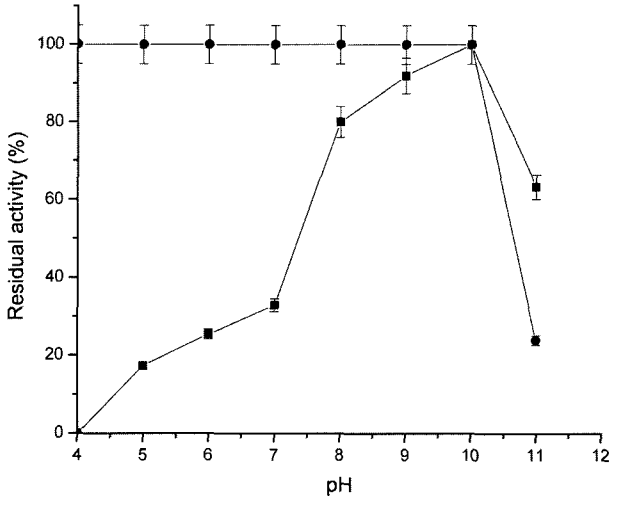

Fig. 3. Effect of pH on β-mannanase activity (■) and stability of the enzyme (●).

xylanase from *B. thermoalkalophilus* had a half-life of 150 min at 60°C [17]. The use of thermostable β-mannanase from *Bacillus* sp. is more advantageous in the pulp and paper industry for biobleaching of Kraft pulp than that of the same enzyme from mesophilic counterparts.

The influence of pH on enzyme activity is shown in Fig. 3. The enzyme exhibited maximum activity at pH 10, and was found to be active between pH 8.0 to 10.5. As far as stability was concerned, it retained 100% of its original activity upto pH 10.0, whereas it lost 70% to 80% of its original activity at pH 10.5 and 11, respectively. In the earlier reports on β-mannanase, only the enzyme from *Bacillus* sp. possessed a higher optimum between pH 8.0

Table 3. Effect of some ions and inhibitors on enzyme activity.

Metal ions/Inhibitors	Enzyme activity (U/ml)
Control	100.00
$MnSO_4$	18.75
$MgSO_4$	94.28
$HgCl_2$	6.98
$CuSO_4$	8.55
$ZnSO_4$	63.56
$CoCl_2$	68.59
$FeCl_3$	73.16
$CaCl_2$	105.72
Cysteine hydrochloride	29.26
$AgNO_3$	0.00
N-Bromosuccinamide	8.55
1,10-Phenanthroline	19.98
EDTA	18.28
PMSF	22.86
DTT	23.60

Each value is the mean of three assays.

to 10 [7], and a greater stability at pH 5.0 to 10.0 [5]. β-Mannanase from thermophilic bacterium *Rhodothermus marinus* exhibited optimum activity between pH 5.0 to 6.5 [6], and the halotolerant strain NN exhibited optimum activity at pH 7.6 [18].

The response of the enzyme activity to different metal ions and inhibitors varied greatly (Table 3), which was confirmed by triplicate readings. The β-mannanase activity was enhanced in the presence of calcium chloride and ferrous sulfate. $AgNO_3$ completely inhibited the activity, and significant loss in the activity was observed in the presence of manganese, mercury, and copper salts, which are believed to be the oxidation of amino acid residues essential for the enzyme activity. In similar earlier reports, Hg^{+2} ions completely inhibited the enzyme [14]. $HgCl_2$ and $AgNO_3$ strongly inhibited the enzyme from *Bacillus* sp. [7].

The loss of activity in the presence of chelating agents such as EDTA and 1,10-phenanthroline suggests that the enzyme is divalent and cation-dependent, and tryptophan is likely the candidate present at or near the active site of the enzyme [13]. The crucial role of tryptophan in β-mannanase activity was additionally supported by the strong inhibition by N-bromosuccinimide, which was also true in the case of mannanase from *Streptomyces* sp. [10]. When dithiothreitol was used, a 75% fall from its maximum activity was noticed, suggesting that the antioxidant compounds break the disulfide (-S-S-) bonds and in turn bring down the activity. The presence of PMSF, an agent that oxidizes -SH groups, resulted in an 80% loss of the enzyme activity, which suggests the involvement of -SH groups in the hydrolyzing activity of β-mannanase. The enzyme activity was also inhibited by EDTA, urea, and SDS, and similar observations have been reported by Jigyodan and Shusei [7] with regard to *Bacillus* sp.

This organism has multiple utility, producing not only thermostable alkaline β-mannanase but also cellulase-free thermostable alkaline xylanase [8]. The combined action of cellulase-free xylanase and β-mannanase has great significance for pulp bleaching in the paper industry [3]. This ecofriendly enzyme, which is active in alkaline conditions (pH 7.5 to 9.0) and higher temperatures, is preferred for the hydrolysis of polysaccharides in the pulp and paper industry. For further industrial-scale enzyme production, scale-up strategies using inexpensive carbon and nitrogen sources will have to be targeted.

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