SOLID STATE FERMENTATION AND CHARACTERIZATION OF PARTIALLY PURIFIED THERMOSTABLE MANNANASE FROM *Bacillus* sp. MG-33

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Bacillus sp. MG-33 was isolated from the desert of Rajasthan (India). The organism produced 500 and 200 Ug^{-1} of thermostable mannanase (after 96h) in solid state fermentation (SSF) of wheat bran and wheat straw rich-soda pulp at the moisture ratio of 1:1.5 and 1:3 at 30ºC, respectively. Two-step partially purified mannanase was optimally active at 65ºC and was 100% thermostable at 55 to 60ºC for 2h and also retained more than 50% residual activity at 65ºC for 2h. A pH of 6.5 was optimum for enzyme activity and 100% stability up to 4h at this pH. Mannanase activity was slightly enhanced by Ca^{2+}, Fe^{3+}, and Mg^{2+}, while 100% activity was retained in the presence of Ba^{3+}, Li^{+}, and $NiCl_2$ at 1.0-10mM. 1M NaCl and urea did not reduce the enzyme activity. The K_m and V_{max} of mannanase were 0.2mgml^{-1} and 60U$mg^{-1}ml^{-1}$, respectively. Hydrolysis of locust bean was rapid and linear between 5 and 20 min, and ~300µgml^{-1} mannose was obtained after 20 min of catalytic reaction by enzyme at 65ºC. TLC was used to confirm the mannose as an end product after hydrolysis of locust bean gum.

Key words: Bacillus sp. MG-33; Mannanase; Solid state fermentation; Thermophilic

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INTRODUCTION

Mannan exists in nature in two forms, galactomannan and acetylated galactomannan. Galactomannan is present in the seeds of leguminous plants and composed of a homogenous backbone of β-1-4 linked mannose residues. Acetylated galactomannan, a principal component of hemicellulose, has a heterogenous backbone of β-1-4 linked mannose and glucose units. Mannanases (EC 3.2.1.78; 1,4–β- D-mannan mannanohydrolases) occur widely in microorganisms in fungi, yeasts, and bacteria as well from germinating seeds of terrestrial plants (Ferreira and Filho 2004; Heck et al. 2005; Jiang et al. 2006; Lin et al. 2007). Production of mannanase by microorganisms is more promising due to its low cost, high production rate, and readily controlled conditions. Mannanases are enzymes useful in several industrial processes such as extraction of vegetable oils from leguminous seeds and the reduction of viscosity of coffee extracts during the manufacture of instant coffee. In the paper industry mannanases have synergistic action in biobleaching of the wood pulp, significantly reducing the amount of chemicals used (Khanongnuch et al. 1998).

Currently it is important to minimize the use of chlorine-based chemicals used in bleaching of pulps. The available options include oxygen delignification, extended cooking, and substitution of chlorine dioxide for chlorine, hydrogen peroxide, and ozone treatments. But most of these methods involve high capital investment for process changes. Thus there is a need for alternatives and cost-effective methods. Among these, biobleaching with enzymes has shown immense potential in minimizing use of bleaching chemicals containing chlorine (Ninawe and Kuhad 2006; Singh et al. 2008). The use of biotechnology in pulp bleaching has attracted considerable attention and achieved interesting results in recent years. The growing interest in mannanase production for industrial applications is due to its importance in the biotransformation of agro-industrial residues.

In the present study mannanase production was observed in solid state fermentation of wheat bran and wheat straw rich-soda pulp by *Bacillus* sp. MG-33. In order to develop an efficient mannan degradation process, knowledge regarding environmental factors affecting the solid state fermentation by newly isolated *Bacillus* sp. MG-33 has to be observed and the enzyme also needs to be partially purified and characterized.

EXPERIMENTAL

Solid State Fermentation of Wheat Bran and Wheat Straw Rich-Soda Pulp

In solid state fermentation experiments wheat bran was primarily used as the solid substrate. 10g of bran was thoroughly mixed with mineral salt solution at different moisture levels of 1:11 to 1:35. Later on all flasks were inoculated with 1% of overnight grown seed culture and incubated at 30ºC for 120h. One gram of spent solid wheat bran was withdrawn after every 24h up to 120h and then suspended in 10mL of citrate phosphate buffer (100mM) at pH 6.5, and then it was vortexed thoroughly. Then the solution was centrifuged at 10,000 rpm for 20 min at 5ºC to recover all mannanase activity in the supernatant. Thereafter the enzyme yield was determined. The same procedure was followed as for the fermentation of wheat straw rich-soda pulp.

Composition of Minimal Media (Mineral Salt Solution)

The minimal media composition was Na_2HPO_4 0.7%, KH_2PO_4 0.3%, NH_4Cl 0.1%, NaCl 0.5%, soil extract 2ml (v/v), and the pH was adjusted to 7.0 with 10% Na_2CO_3.

Enzyme Assay

Mannanase activity was determined by using locust bean gum (0.5% w/v) as the substrate. The mannanase activity was measured in terms of the amount of reducing sugars released from locust bean gum by action of enzyme. Reducing sugars were measured by using the 3,5-dinitrosalicylic acid (DNSA) method of Miller (1959). The amount of sugars released (mg ml^{-1}) was determined by using mannose as a standard. One international unit of mannanase activity was defined as the amount of enzyme that is required to release the 1 µmole of mannose under standard (pH 6.5 and temp. 65ºC) assay

conditions. Mannanase activity was expressed in terms of Uml^{-1}. Protein was measured by Lowry's method (1951).

Purification of Mannanase

Bacillus sp. MG-33 was cultivated in production media (pH 7.0) having 0.5% locust bean gum and 0.5% yeast extract, under shaking conditions (150 rpm) at 30ºC for 72h (Bhoria et al. 2009). Cell-free supernatant was saturated with 50% ammonium sulphate at 5ºC and was left for 3h to assure a proper precipitation of protein. Precipitates of protein were separated by cold centrifugation at 10000 rpm for 30 min and later dissolved in a minimal amount of citrate phosphate buffer, pH 6.5. The dissolved protein precipitates were dialysed against same buffer for 15-24h. Completely dialysed protein was then loaded on the sephadex G-150 column. Before loading, the sample matrix was washed thrice with citrate phosphate buffer of pH 6.5. The flow rate of the column was 25 mlh^{-1}.

Effect of pH on Mannanase Activity

The optimum pH for mannanase was determined at 65ºC by assaying the enzyme in the pH range of 3.0 to 10 (citrate phosphate, pH 3.0-7.0, tris-malate, pH 7.0-9.0, phosphate buffer, pH 6.3-7.8, and glycine-NaOH buffer, pH 8.6-10.5.

pH Stability of Mannanase

To observed the pH stability, mannanase samples were kept with different pH buffer(s), ranging from 5.0 to 8.0 at room temperature for different time intervals up to 4h. Thereafter the enzyme activity was assayed.

Optimum Temperature and Thermostability of Mannanase

The optimum assay temperature for mannanase was determined by keeping the assay mixtures containing mannanase and locust bean gum (pH, 6.5) in the temperature range of 10-100ºC. Thermostability of enzyme was also examined at pH 6.5. Mannanase samples were incubated at various temperatures (55-75ºC). The samples were withdrawn at regular intervals up to 2h, and thereafter residual activities were evaluated under the standard assay conditions.

Hydrolysis of Locust Bean Gum by Mannanase from Bacillus Sp-MG-33

20 U of mannanase was diluted to 20ml, then incubated with 1% w/v locust bean gum in citrate phosphate buffer, pH 6.5 at 65ºC. A 0.5mL aliquot of sample was withdrawn from the reaction mixture at regular intervals (0-60 min). The rate of hydrolysis of the locust bean gum was determined by measuring the amount of reducing sugars released, using the method of Miller (1959).

End Product Analysis (mannanase treated locust bean gum) by Thin Layer Chromatography (TLC)

TLC was performed by the method of Lopez et al. (1998). The reaction between mannanase and locust bean gum was carried out at 65ºC and pH 6.5. The samples were withdrawn at regular time intervals and spotted on TLC plates. The solvent system used

was chloroform:glacial acetic acid: water (6:7:1, v/v). After running the samples, the plates were dried and visualized by spraying them with a solution of ethanol and sulphuric acid (95:5 v/v) after heating the plate at 100ºC for 10 min. The standard used for TLC was mannose.

Effects of NaCl and Urea on Mannanase Activity

The effects of urea and NaCl on mannanase activity were determined by incubating the enzyme with different conc. (0.5-6M) of salt and urea at room temperature for 1h. Thereafter, residual activities were determined under the standard assay conditions.

Estimation of K_m and V_{max} of Mannanase

The kinetics of purified enzyme was studied by using locust bean gum as a substrate variable (conc. 0.25-10mg ml^{-1}). K_m and V_{max} were calculated by line weaver-Burk plot.

Effects of Metal Ions and Organic Compounds on Enzyme Activity

Effects of different metal ions and organic compounds were analysed by incubating the enzyme with metal ions and organic compounds (1-10mM) at room temperature; later the residual activity was measured by the standard assay procedure.

RESULTS AND DISCUSSION

Identification and taxonomical studies of the isolate were carried out according to the standard techniques and protocols mentioned in Bergey's Manual of Systematic Bacteriology (Sneath 1994). The organism was Gram positive and appeared rod-shaped under the compound microscope at 100X resolution. Partial 16S rDNA sequence analysis of this organism will be performed to find out its genus or closest species. All experiments were carried out in triplicates at least. The results presented are mean values. The relative standard deviations were in the range 1.4 to 4.3% ± of the average values.

Solid State Fermentation of Wheat Bran and Wheat Straw Rich Soda Pulp

Results from the production of mannanase confirmed that SSF is a highly attractive process for mannanase production using agro-industrial byproducts. The mannanase production at an industrial level is still limited, although it is known that these are being used in bio-bleaching of various pulps, which reduces the use of chlorine for the lignin removal process. In solid state fermentation, when using wheat bran as a primary substrate, maximum mannanase production was observed to be 500 Ug^{-1} of substrate after 96h of growth at 30ºC with a moisture ratio of 1:1.5 (Fig. 1). In case of solid state fermentation of wheat straw rich-soda pulp the production was 200 Ug^{-1} of enzyme at a moisture ratio of 1:1 within 96h at 30ºC (Fig. 2). Increased mannanase production in wheat bran can be attributed to its content of more enriched and easily biodegradable ingredients. Less production of enzyme in pulp fermentation may be due to less nutrition availability and to the fact that the pulp itself is a little resistant to the microbial attack and contained lignin also. In the present study it was clearly understood

that the moisture level was very crucial, and more enzyme was produced only at a specific moisture ratio. Mannanase is a hemicellulase enzyme and less studied by solid state fermentation as compared to other hemicellulases enzymes. SSF with *bacillus* sp. MG-33 yielded greater production of mannanase in both cases (SSF of wheat bran and pulp) at a low moisture level of 1.15 for wheat bran and 1.11 for wheat straw rich-soda pulp, which may be due to the xerophilic habitat of the organism, because *Bacillus* sp. MG-33 was isolated from the desert.

However, the 1:1 moisture ratio was specific for the production of xylanases from *Bacillus* sp. A-09 (Gesesse and Mamo 1999), and a maximum amount of amylase was produced at a 30% moisture level (Baysal et al. 2003). Maximum xylanase yield was obtained from *Streptomyces* sp. QG-11-3 in solid state fermentation of wheat bran and eucalyptus kraft pulp at a substrate to moisture ratio of 1:3 and 1:2.5 respectively (Beg et al. 2000). The present study is also important for the chlorine-free bleaching of pulps, because mannanase itself is a hemicellulase enzyme, and it has been reported as a cause of delignification of kraft and non-woody pulps (Sunna et al. 2000; Kanosh and Nagieb 2004). It was not clearly understood why mannanase production was reduced after 96h of incubation of all culture moisture levels studied with the two substrates. This may be due to release of total protease after 96h due to auto-lysis of cells, which may have initiated the degradation of mannanase after 96h. Solid state fermentation of wheat straw rich-soda pulp gave 200 Ug^{-1} of mannanase and gave a new possibility for direct use of *Bacillus* sp. MG-33 in bio-pulping/ biobleaching, for weakening the bond strength between lignin and hemicellulose.

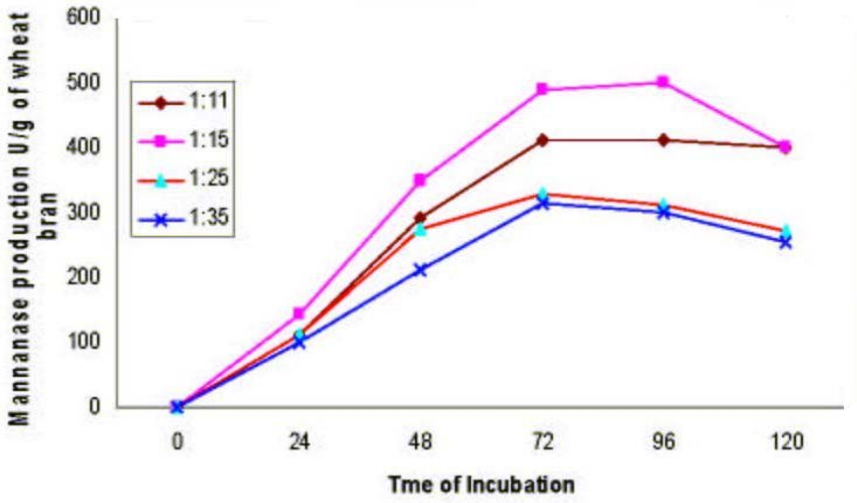

Fig. 1. Effect of moisture ratio in solid state fermentation (wheat bran) by *Bacillus* sp. MG-33 at pH 7.0 and 30ºC under static conditions

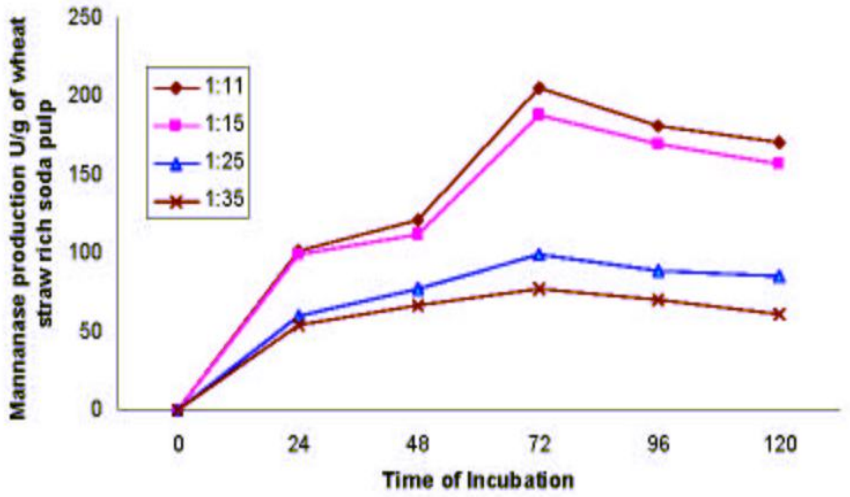

Fig. 2. Effect of moisture ratio in solid state fermentation (wheat straw rich-soda pulp) by *Bacillus* sp. MG-33 at pH 7.0 and 30ºC under static conditions

Purification of Mannanase from Bacillus sp. MG-33

Two-step purification of mannanase was performed, and an 11.8-fold purification was achieved with 591 Umg^{-1} specific activity (Table1). After 50% ammonium sulphate saturation of protein broth, the precipitates were dialyzed against 100mM citrate phosphate buffer (pH 6.5) for up to 15h at 4ºC. Dialysed protein was loaded onto a sephadex G-150 column, which was eluted with 100mM citrate phosphate buffer (pH 6.5). Mannanase activity was detected in fractions 17 to 27. These collected fractions were pooled and later used in characterization of the enzyme.

Table 1. Two-step Purification of Mannanase by Ammonium Sulphate Saturation and Sephadex G-150

Purification steps	Volume (ml)	Total mannanase activity	Total protein $mgml^{-1}$	Specific activity Umg^{-1} protein	Purification fold
Cell free supernatant	250	6250	175	35.7	1.0
Ammonium-sulphate(50%)	50	2000	40	50	1.4
Sephadex-G-150	18	1000	1.69	591.7	11.8

Characterization of Mannanase

Effect of pH on activity and stability of mannanase

The enzyme was maximally active at pH 6.5, whereas there was almost a complete loss of activity at pH below 4.0 and above the 9.0 pH. Stability of the enzyme was checked at different pH values ranging from 5.0 to 8.0 and incubating the samples at room temperature for 4h. Thereafter, residual activity was determined at 65ºC. As shown

in Fig. 3 at pH 6.5, the enzyme was completely stable without loss of any activity at room temperature after 4h of incubation. The optimum pH values for mannanase from *Bacillus licheniformis* (Zhang et al. 2000) and *Streptomyces lipomoea* (Montiel et al. 2002) have been determined to be 7.0 and 7.5, respectively. Akino et al. (1987) reported that mannanase from alkalophilic *Bacillus* sp. had pH optima of 9.0 and were stable between pH values of 8.0 and 9.0 but became progressively unstable at pHs below 8.0.

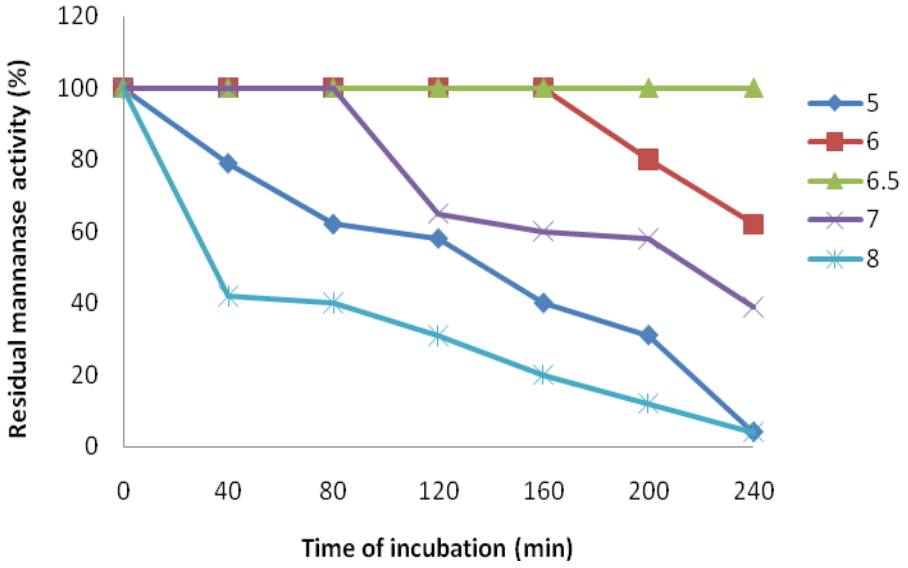

Fig. 3. Effect of pH on mannanase stability at temperature 65ºC (100% activity = 50Uml^{-1})

Temperature Optima and Thermostability of Mannanase

The optimum temperature for enzyme activity was determined by varying the reaction temperatures between 10 and 100ºC. Mannanase activity was 100% expressed at 65ºC. To examine thermal stability, the enzyme was kept at different temperatures (55 to 75ºC) for various time intervals. The residual activity was measured at 65ºC at pH 6.5 (Fig. 4). Mannanase was maximally stable at 55 ºC to 60 ºC and retained activity (~100%) for 2h. A higher temperature optimum of 70 ºC has been reported for mannanase from *Penicillium purpurogenum* (Park et al. 1987). A higher temperature optimum and temperature stability of mannanase from *Bacillus sp.* MG-33 makes it a more suitable catalyst for possible application in biobleaching of pulps at higher temperature and also for use in biopulping, where the temperature of the process can be held within the range 80 to 115 ºC.

Hydrolysis of Locust Bean Gum by Mannanase

The rate of locust bean gum hydrolysis by mannanase was rapid and linear during the period 5 to 20 min, and thereafter it followed a stationary patteren (Fig 5). Results from the hydrolysis study showed that this enzyme has a strong potential to be use in sugar industries for the production of mannose from mannan by enzymatic hydrolysis without causing any pollution as compared to the use of toxic inorganic acids in preparation of mannose from mannan by acid hydrolysis.

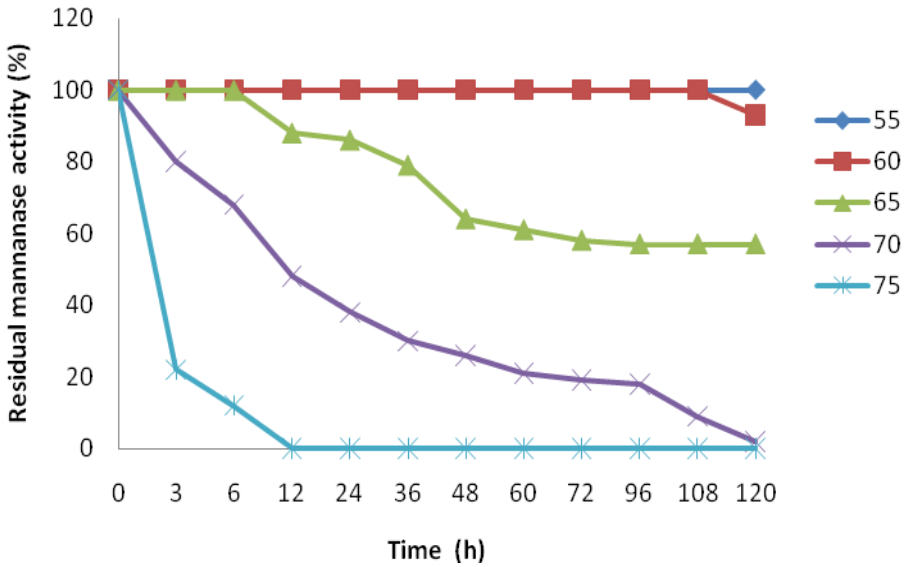

Fig. 4. Thermostability profile of mannanase at pH 6.5. (100% activity= 50Uml^{-1})

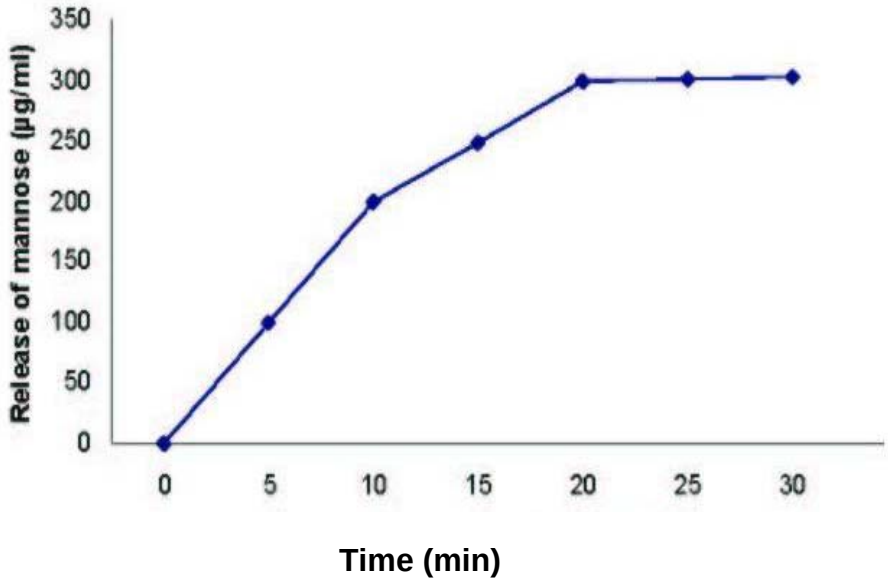

Fig. 5. Hydrolysis of locust bean gum by mannanase from *Bacillus* sp. MG-33, at pH 6.5 and 65ºC

Thin layer Chromatography

The hydrolysis products from locust bean gum were analyzed by thin layer chromatography, using silica gel. Mannose was found to be the prominent end product formed after 90-minutes of incubation with purified enzyme (Fig. 6).

Fig. 6. End product analysis of locust bean gum (mannan) after hydrolysis by mannanase from *Bacillus* sp. MG-33 (Lane A: Controll sample of mannose 10µg/ml, Lane B: Hydrolysis extract of locust bean gum and mannanase.

Stability of Mannanase in High Concentration of Urea and NaCl

Mannanase from the *Bacillus* sp. Mg-33 tolerated 1M urea and 1M NaCl (Figs. 7 and 8) concentration without any significant loss of enzyme activity. Further, mannanase showed its >70% and 60% activity up to 4 and 3M concentration of urea and NaCl, respectively. This resistance of the enzyme to NaCl is a desirable factor relative to enzyme use in biobleaching of pulps where the Na^+ and Cl^- ion concentrations can be very high. On the other hand, the urea stability also makes it suitable for use in agriculture waste (lingo-cellulose) bio-degradation where the urea concentration is often very high because it is used as an organic fertilizer.

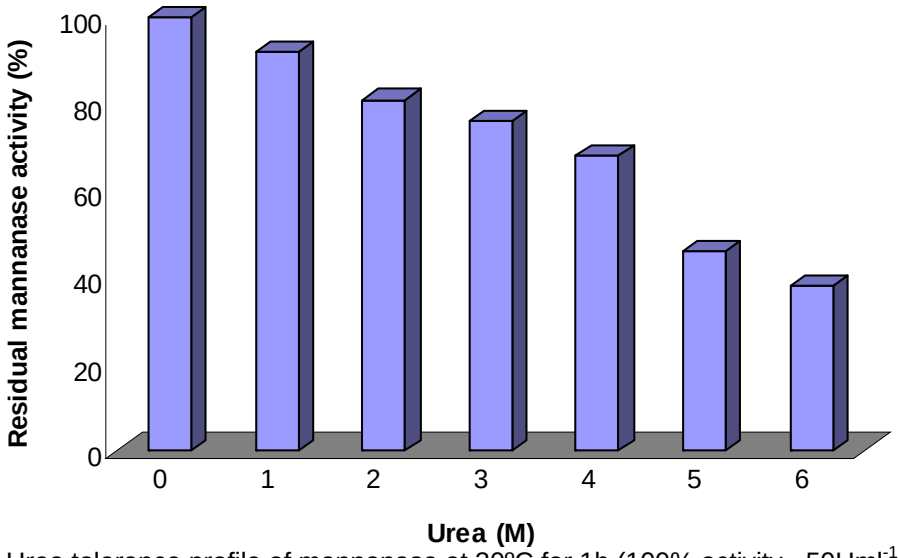

Fig. 7. Urea tolerance profile of mannanase at 30ºC for 1h (100% activity= 50Uml^{-1})

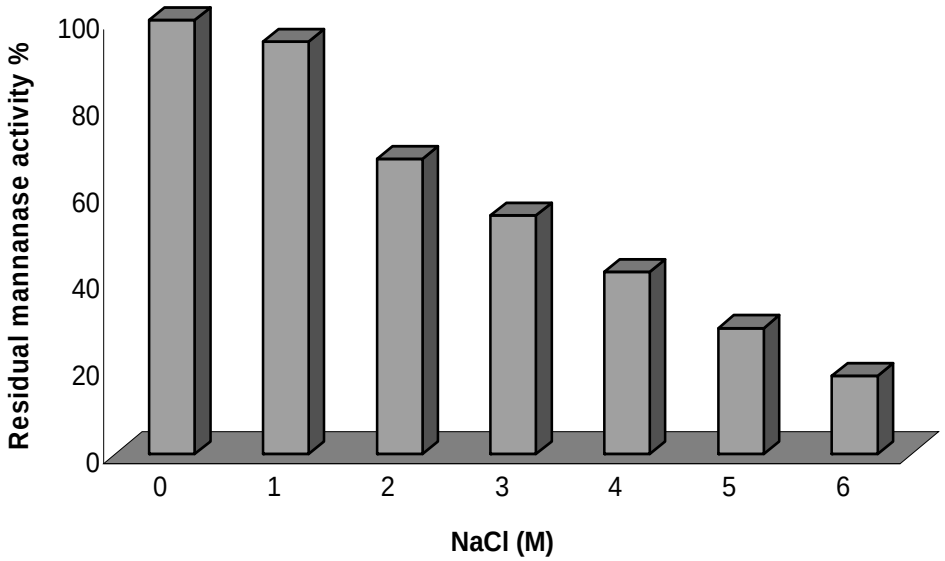

Fig. 8. NaCl tolerance profile of mannanase at 30ºC for 1h (100% activity= 50Uml^{-1})

Table 2. Effect of Metal Ions and Organic Compounds on Mannanase Activity from *Bacillus* sp. MG-33

Metal ions/Organic -compounds	Residual activity (%)	
	1 mM	10 mM
Control	100	100
$AgCl_2$	68	68
$BaCl_2$	100	100
$CaCl_2$	120	115
$CdCl_2$	70	70
Fe^{++}	130	120
Fe^{3+}	90	90
$HgCl_2$	0.0	0.0
KCl	115	110
$LiCl_2$	100	110
$NiCl_2$	90	85
$MgCl_2$	125	100
$CuCl_2$	20	13
EDTA	90	23
TritonX-100 (1% v/v)	90	90
SDS (0.1% w/v)	0.0	0.0
H_2O_2 (0.1% v/v)	55	0.0
Bleaching powder (0.1% w/v)	65	0.9

100% activity = 50 Uml^{-1}

Determination of K_m and V_{max}

The mannanase from *Bacillus* sp. MG-33 exhibited a k_m value of 0.2 mg ml^{-1} when the substrate used was locust bean gum. V_{max} of this enzyme was 60 U min^{-1} mg^{-1} protein. The mannanase from *Bacillus stearothermophilus* showed K_m and V_{max} values of 1.5 mg ml^{-1} and 455 Umg^{-1} protein (Talbot et al. 1990), whereas Montiel et al. (2002) reported K_m and V_{max} values of 3.4 mg ml^{-1} and 55.7 Umg^{-1}, respectively.

Effect of Metal Ions and Organic Compounds on Mannanase Activity

Mannanase activity was slightly stimulated by Ca^{2+}, Fe^{3+}, and Mg^{2+} at concentrations between 1 and 10 mM (Table 2). Also, the enzyme retained its full activity in the presence of Ba^{3+}, Li^{+}, and $NiCl_2$ (1 to 10mM). On the other hand, Hg+ was found to be 100% destructive to mannanase activity at both concentrations 1-10 mM. No organic compound was found to enhance the enzyme activity, although in the presence of EDTA the enzyme showed 90% activity. Montiel et al. (2002) reported the complete inhibition of mannanase by Hg^{+} ions. The effect of chloride salts on mannanase activity was tested, and results showed few instances of enhancement of the catalytic activity, but in the presence of some salts enzyme retained 100% of its activity. This behaviour of mannanase is not understood.

CONCLUSIONS

1. Mannanase was produced from a new *Bacillus* sp. MG-33 strain by solid state fermentation of wheat bran and wheat straw rich-soda pulp.
2. The partially purified mannanase was characterized by evaluating its activity and stability under various unfavorable physical and chemical conditions.
3. Results indicated that the enzyme is resistant to various metal salts and it has potential to be used in biobleaching of pulps and in bio-pulping, where various metal ions are present in excess, especially NaCl.

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