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RESEARCH ARTICLE OPEN ACCESS

Purification of a Thermostable β-mannanase from *Paenibacillus thiaminolyticus* - Characterization and its Potential Use as a Detergent Additive

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Abstract

Endo-1, 4- β- D-mannanase (EC 3.2.1.78) is a glycoside hydrolase involved in random cleavage of β-1, 4- D-manno-pyranosyl linkages within mannans and heteromannans and generates branched and linear oligosaccharides. A β-mannanase was purified from a thermotolerant bacterium *Paenibacillus thiaminolyticus* isolated from a soil sample. Enzyme was purified to homogeneity with specific activity of 8812 U/mg protein. Sodium dodecyl sulfate (SDS) and native poly-acryl amide gel electrophoresis indicated that the purified mannanase is a monomeric protein with a molecular mass of 38 kDa. The purified enzyme was found to be maximally active at temperature and pH of 60°C and 7.0, respectively. It was stable at 55°C for 24 h and maintained more than 50 % activity up to 3 h at 60°C. The enzyme was very stable in the pH range of 5.0-9.0. Purified β-mannanase demonstrated high stability after 1 h of pre-incubation with most of the tested organic solvents. Enzyme retained significant stability in the presence of various detergent additives, commercially available detergents and dish washing liquids. The high compatibility and substantial stability in the presence of nonionic detergents and dishwashing liquids confirmed its utility as an additive to dish washing liquids and laundry detergents. Enzyme exhibited efficacious de-staining of heteromannan based stains of chocolate ice cream and salad dressing in the wash performance test for detergent application. It also exhibited anti-soil redeposition effect on cotton swatches treated with tennis court clay and heteromannans.

Keywords: Endo-1, 4-β-D-mannanase, heteromannan, dishwashing liquid, detergent additive, purification, wash performance

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INTRODUCTION

Mannans and heteromannans are natural polysaccharides existing as part of the hemicellulose fraction in plant cell walls[1]. Endo-1, 4-β-D-mannanase (EC 3.2.1.78) is an endohydrolase, which randomly cleaves pyranosyl linkages within the main chain of various heteromannans and mannan to release oligosaccharides of different lengths. β-mannanase production is elaborated by diverse grups of bacteria, actinomycetes, and fungi isolated from natural sources[2]. Although a number of mannanase-producing microbial sources are available, only a few are commercially exploited as wild or recombinant strains, of these, the important ones are: *Trichoderma longibrachiatum, Aspergillus niger, Bacillus* sp., thermophilic microorganisms from hydrothermal vents[2]. In spite of several available reports on mannolytic microorganisms, their practical potentialities were yet not fully exploited[3]. β-mannanase has been studied mainly with respect to their varied industrial potential[4]. A bacterium *Paenibacillus thiaminolyticus* was isolated in our laboratory and reported previously because of its rapid growth, capacity to secrete a high level of extracellular β-mannanase (1100 ± 50U/ml), into the growth medium and for its potential prebiotic properties[5]. During the course of study, on the application of enzyme, another possible use for mannanase as an additive for improving the efficacy of detergents was evaluated. Very few reports exist on detergent application studies of β-mannanase[3,6]. Ideally, for an enzyme to be used in detergent formulation, it should exhibit stability over a broad range of temperature and pH. Thermal stability in the presence of detergent components such as surfactants and various detergent additives is a major prerequisite.

In the present study, thermostable β-mannanase from *P. thiaminolyticus* was purified and characterized. The efficacy of the enzyme was examined for its wide application in detergent industry. Previous studies on purification and characterization of β-mannanase had been reported from *Paenibacillus* sp. BME-14[7], *Paenibacillus* sp. DZ3[8], *Paenibacillus* sp. CH-3[9], but its potential as detergent additive had not been explored.

MATERIALS AND METHODS

Microorganism and culture conditions

A thermotolerant bacterium *Paenibacillus thiaminolyticus* producing an extracellular thermostable β-mannanase isolated from a soil sample was reported earlier[5]. Optimized production medium used was a Mineral salt (MS) medium complemented with 2 % WB (wheat bran) adjusted to pH 7.0. The mineral salt medium was inoculated with 16 h seed culture (2 % inoculum) and incubated for 48 h at 50°C. The cell pellet was separated by centrifugation at 6000×g for 10 minutes in a cooling centrifuge and cell-free clear supernatant was used as crude enzyme[5].

Materials

Mannan (Ivory nut) and Manno-oligosaccharide were acquired from Megazyme. Konjac Gluco Mannan (KGM) was obtained from TerraVita Herbs, Ontario. Various column media and columns used were procured from Pharmacia. All chemicals used were of analytical grade.

Assay for mannanase activity and protein assay

Enzyme activity was assayed using cell free clear supernatant. β-mannanase activity was assayed by the dinitrosalicylic acid (DNSA) method of Miller[5,10]. Protein concentration was measured by the method of microbicinchoninic acid (BCA) protein assay using a protein assay kit. BSA- bovine serum albumin was used as the standard. All column chromatographic elutes were monitored for protein content by recording absorbance at 280 nm. All the experimental values represent the mean of three determinations carried out in duplicate. In no case did the difference between duplicates exceed 5%.

Purification of β-mannanase produced by *P. thiaminolyticus*

β-mannanase was purified from extracellular fraction using multiple steps. The purification procedure was simplified by the presence of low amount of total protein in the extracellular fraction as compared to cell extract. To the clarified pre-chilled culture supernatant ammonium sulphate was added with constant stirring to achieve 80% saturation. The mannanase activity and the protein content were determined both in the pellet and the supernatant. β-mannanase activity was recovered in the pellet,

pellet was resuspended in 0.05 M potassium phosphate buffer (KPB)-pH 7.0. Mannanase fraction obtained was subjected to hydrophobic interaction chromatography. Mannanase fraction obtained was loaded on a pre equilibrated Phenyl Sepharose (Pharmacia) column (9.0 X 1.0 cm2) with equilibration buffer. Active fractions with mannanase activity were pooled together and the volume of pooled fractions was noted. Protein concentration as well as enzyme activity was determined for the fractions. Dialyzed pooled HIC-fraction containing mannanase was concentrated on an Amicon ultrafiltration membrane filter with 10 kDa cut-off. The fractions were analyzed for purity and mannanase activity[5].

SDS-PAGE and Zymogram

Molecular weight of the purified enzyme was estimated by performing SDS-PAGE in a 12.0 % (w/v) polyacrylamide gel. Pre-stained medium range (14.3–66 kDa) molecular weight markers were used. Coomassie brilliant blue R-250 staining was done to observe protein bands.

Zymogram was obtained by modifying the method of Blank et al.[11]. After electrophoresis, gel was soaked in 0.1% (v/v) Triton-X 100 with gentle shaking to remove SDS and to renature enzymes in the gel. This washing process was repeated twice for 30 min. Final washing was given in 100 mM KPB (pH 6.5) with gentle shaking for 15 minutes. To detect mannanase activity, gel was placed on an agarose mannan sheet (10 mm thick) that included 1% glucomannan, 1.8% agarose, and 100 mM KPB (pH 6.5) and incubated at 55°C for 45 min[12]. The polyacrylamide gel was separated off from the mannan agarose gel sheet. Agarose gel sheet containing substrate was stained with (0.1 %, w/v) congo red solution.

Characterization of purified mannanase

The optimal temperature for purified enzyme preparation was analysed at different temperature ranging from 37-80°C. Purified mannanase thermal stability was assayed in temperature ranges of 55 to 80°C. The time of incubation varied from 30 to 120 minutes. Aliquots were withdrawn periodically to assay residual enzyme activity at pH 7.0 and at 60°C. To determine the optimum pH of purified enzyme, its activity was assayed at different pH from (4.0-12.0) at 60°C. For pH 4.0-12.0 (50 mM) Citrate Buffer, Phosphate Buffer, Tris-Buffer, Glycine-Sodium Hydroxide Buffer were prepared. To test the pH stability, equal volume of the purified mannanase was incubated with various buffers mentioned above (50 mM) ranging from pH 4.0-10.0 at 55°C for 1 h to overnight. The residual enzyme activity was subsequently determined using DNS assay as described before. The unincubated sample served as control (100% activity).

Substrate specificity and kinetic analysis

Substrate specificity of the purified mannanase was determined by incubating the enzyme with one of the following substrates (1% w/v): Locust bean gum, Xylan (oat spelt), Mannan (ivory nut), Starch, Guar gum, Pectin, Chitin, Konjac glucomannan, and CM-cellulose prepared in potassium phosphate buffer (50 mM)-pH 7.0. Reducing sugar liberated from different polysaccharides was quantified under standard assay conditions in the reaction product.

Kinetic constants, *Vmax* and *Km*, were determined for β–mannanase by using Lineweaver-Burk plot assuming that simple Michaelis-Menten kinetics was followed. The mannanase activity was calculated at its previously determined optimal pH and temperature. The *Vmax* and *Km* for purified enzyme were evaluated by varying concentrations of the LBG (0.1 -10 mg/ml) as substrate.

Effect of stabilizers/ organic solvents

The significant effect of different organic solvents ethanol, methanol, propanol, DMSO, glycerol, propanone, ethylene glycol, polyethylene glycol and isoamyl alcohol on enzyme stability was determined at 30% (v/v) concentration. Residual β–mannanase activity was quantified by incubating purified enzyme for 60 min to overnight with the selected organic solvent at 55°C.

Suitability of β-mannanase for detergent application

Effect of surfactants/detergent additives

Stability and compatibility of *P. thiaminolyticus* purified mannanase with various ionic (negatively charged-SDS and positively charged-CTAB and non-ionic detergents- Polysorbate-20 (PS-20), Polysorbate-40 (PS-40), Triton-X-100 (TX-100) were checked. β-mannanase was incubated in the detergents at 60°C for one hour. The stability of the mannanase in various detergent additives [builders (zeolites, sorbitol, mannitol, citric acid), bleaching agents (H_2O_2, sodium perborate) and other components like optical brighteners

(Polyethylene-glycol), anti-redeposition and soil suspension agent (CM-cellulose), dye tranfer inhibiting agents (Polyvinyl pyrollidone) and bulking agent (Sodium sulfate)] was investigated by incubating 0.5 U/mL mannanase with these agents (7% w/v) for 60 min at 55 C. Residual β–mannanase activity was quantified in the samples treated with different detergent additives. Enzymatic activity determined in the absence of any surfactant/ detergent additives under similar conditions was taken as control.

Compatibility of mannanase with commercial detergents

Effect of various locally available detergent powders was replicated by testing the stability of the purified mannanase in the presence of detergents powders of different compositions viz. Rin, Ariel, Wheel, Tide, Henko, Nirma, Ranipal and Ezee, and dish washing Liquids viz. Pril, Scrubz, Vim and Dettol Dish. The assay for mannanase was carried out in the presence of these washing powders/liquids, by adjusting the conc. of each detergent to 0.7% in a final volume of assay mixture, using LBG as a substrate at 60°C. The effect of all washing powders was also checked by adjusting their pH to 7.0, to overcome the effect of variable pH of different washing powders on enzyme activity and stability.

Cleaning performance test

To check efficacy of purified mannanase as a detergent additive, multiple white cotton swatches that were stained with chocolate ice cream and salad dressing dried overnight at 37°C were used. To evaluate the cleaning ability of purified mannanase, stained cotton swatches were soaked in a series of flasks (from a-f), where each flask contained unfiltered tap water (20 ml) and different combinations of detergents and detergent additives with or without enzyme (5 ml) each of :

1. Detergent [Henko] (7 mg ml^{-1})
2. Detergent [Henko] (7 mg ml^{-1}) containing 1U ml^{-1} mannanase,
3. Liquid detergent [Ezee] (1% v/v) containing 1U ml^{-1} mannanase,
4. 1U ml^{-1} mannanase.
5. TX-100 (1%) containing 1U ml^{-1} mannanase,
6. Homemade detergent mix containing non-ionic surfactant TX-100 and all other detergent additives (sorbitol, H_2O_2, PVP, CM-cellulose, PEG) containing 1U ml^{-1} mannanase.

Commercial detergents were heated at 65°C for 15 min to inactivate any inherent enzyme pre-added to detergent to avoid interference with cleaning performance.

To test anti-soil redeposition on fabric, clean cotton swatch was dipped in a set of beakers containing selected detergent mix along with tennis court clay and LBG. The solution was kept in swirling motion at 60°C for 15 min with mannanase and the cotton swatches were taken out to wash. The swatches were rinsed with water, two to three times and then allowed to dry. The cleaning performance was evaluated and compared with a set of control cotton swatches. The control swatches were swirled without adding mannanase in the detergent mix.

RESULTS AND DISCUSSION

Protein purification and molecular weight determination

β-mannanase was fractionated from clear culture supernatant of *P. thiaminolyticus* using salting out by 80% ammonium sulfate

Table 1. Purification of β-mannanase by ammonium sulphate, phenyl sepharose and ultrafilteration from *P. thiaminolyticus* culture filtrate

Purification step	Total Enzyme Activity (U)	Total Protein (mg)	Specific activity (U mg^{-1} of protein)	Yield (%)	Fold purification
Culture filtrate	52164	30	1738.8	100	1
Ammonium Sulphate fraction (80%)	24957	4.45	5608.5	47.8	3.20
Phenyl Sepharose fraction	13951	1.99	7011	26.7	4.03
10 kDa cut off membrane	9515	1.08	8812.8	18.2	5.06

a. Activity was measured in 50 mM KPB (pH 7.0) using 1.0 % (w/v) locust bean gum as substrate by the DNS method.
b. The protein was measured using the method of BCA using BSA (bovine serum albumin) as the standard.

saturation followed by hydrophobic interaction chromatography. Fig. 1 showed the elution of β-mannanase as a single peak from Phenyl Sepharose column. The purification yielded 5.06 fold enhancements in purification and specific activity was 8812 U/mg protein toward LBG (Table 1). Final yield of the purified product was calculated to be 26.7%. Purified mannanase specific activity observed to be quite higher than many bacterial mannanases: 5,065 U/ mg from *Bacillus* sp. N16-5[13]; 4839 µmol/min/mg from *B. subtilis*[14]; 3,590 U/mg from *D. thermophilum* Rt46B.1[15]. Though recombinant mannanases from *B. subtilis* BE-91[16] and *B. subtilis* WY 34[17] demonstrated high specific activity when compared with the mannanase from *P. thiaminolyticus.* Generally highly thermostable endo-β-1, 4-mannanases possess low specific activity related to their mesophilic counterparts[16,18,19]. However, the *P. thiaminolyticus* β-mannanase elucidated in our study is characterized by a particularly high specific activity of 8812 U/mg as well as a relatively high thermostability.

Molecular weight of the purified enzyme was calculated to be 38 kDa. The purified mannanase appeared as a single protein band, corresponding to an active band on zymogram (Fig. 2). A single band as a zone of clearance (yellow halo on a red background) appeared, thus confirming that the purified protein is a mannanase. It further confirmed that this mannanase is a monomeric protein. *P. thiaminolyticus* molecular mass was comparable to those of β-mannanase from *Paenibacillus* sp. DZ3 (39 kDa)[8], and less than that of several *Paenibacilli* sp. (53 kDa, 50.4 kDa)[7,9].

Temperature optima and thermal stability of β-mannanase

The temperature optima for purified enzyme preparation was found to be 60 °C (pH 7.0). The mannanase activity increased upto 60°C but with further increase in temperature it declined. Higher than 70% of the original activity was retained till 75°C but declined to 45% at 80°C. An optimal temperature between 55°C to 70°C was reported from many purified mannanases from *Bacillus* sp.[16,17,20,21].

Thermal stability of purified mannanase was assayed in temperature range of 55 to 80°C. The enzyme showed maximum stability at 55°C, showing no loss of mannanase activity after incubation for 2 h. At 60°C and 65°C the enzyme retained more than 50 % of its original activity after 2 h of incubation. At 70°C and 80°C, half life was 0.38 h and 0.3 h respectively [Fig. 3b]. The residual enzyme activity demonstrated that enzyme was completely stable for 1 h till 60°C.

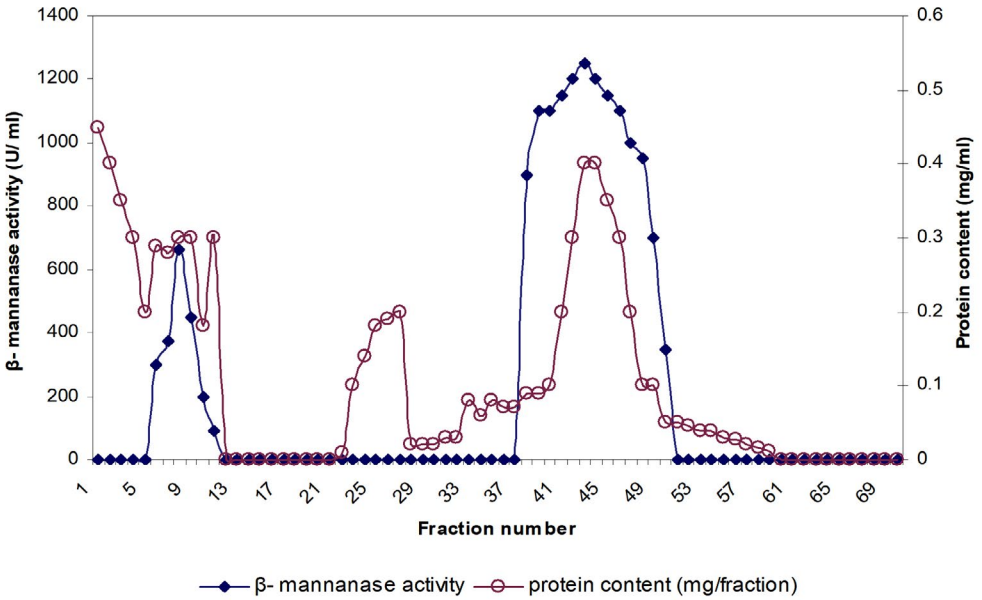

Fig. 1. Hydrophobic interaction chromatography of β-mannanase active fractions, obtained after phenyl sepharose column chromatography. Fractions were tested for protein (—o—) and β- mannanase activity (—■—).

At 70°C the percent residual activity was reduced to 30% after 1 h while only one tenth enzyme activity at 80°C. Residual mannanase activities after 2 h at 60°C and 65°C for *P. thiaminolyticus* were 61.71% and 51.33% respectively. Mannanase revealed analogous thermo stability to the various *Bacilli* strains viz. *Bacillus* sp. SWU60[22] which is stable up to 60°C with 90% residual activity after 1 h. *B. circulans* CGMCC-1554 was stable at 60°C but its activity was completely lost after 20 min of incubation[14]. *P. thiaminolyticus* mannanase possessing higher temperature optima and thermal stability make it a more appropriate candidate for its use in detergent industry.

Effect of pH on activity and stability of β-mannanase

The pH optimum for purified enzyme preparation was pH 7.0 and it demonstrated 90% of maximal activity at a pH range from 6.0 to 7.0. Higher than 65% of the original activity was retained till pH 9.0, whereas enzyme inactivation was observed below pH 4.0. Optimum pH of *P. thiaminolyticus* mannanase was similar to B. subtilis mannanase[22], *B. pumilus* (6.0-7.0), *B. circulans* CGMCC 1416 (5.5-7.0)[14], *B. subtilis* WY 34 (6.5-7.5)[17]. Purified mannanase pH stability was assessed from pH 4.0 to 10.0. The enzyme displayed maximum stability at pH 7.0 retaining 100% of its activity even after 24 h (Fig. 3c). The mannanase exhibited moderate stability in the range of pH 6.0-9.0 at 60°C for 1 h. Purified mannanase possessed higher stability towards slightly alkaline conditions when compared with crude enzyme extracted from *P. thiaminolyticus*[5], Thus, propelling us to explore its possible application in detergents. *Bacillus* sp. SWU60[22] mannanase was stable at pH 5-9 at 4°C for 16 h. *Bacillus subtilis* BM 9602 also showed maximum stability in a range of pH 6.0-8.5[23] which is in agreement with mannanase from *P. thiaminolyticus*.

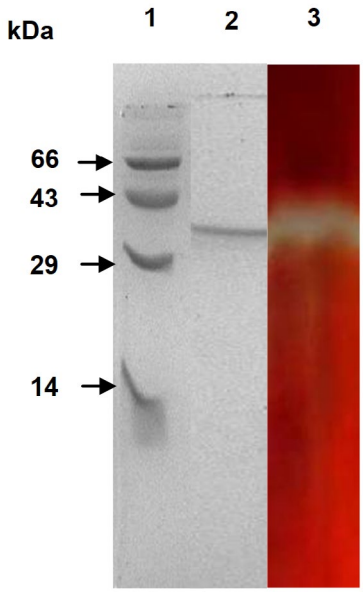

Fig. 2. Analysis of the purified enzyme by SDS-PAGE and zymography. SDS-PAGE (12%) with Coomassie staining. Lane 1- molecular weight markers. Lane 2- The purified enzyme. Lane 3- Zymography with Konjc-glucomannan as the substrate white band indicate β-mannanase activity.

Substrate specificity and kinetic parameters

The activity of purified enzyme towards various substrates was determined. Relative enzyme activity was quantified by measuring the amount of liberated reducing sugars from polysaccharides (Table 2). Among Heteromannans Locust Bean Gum (LBG) released the largest quantity of reducing sugars. Konjac glucomannan (KGM) displayed related results like LBG, when LBG was substituted with Guar Gum (GG) led to approximately 70% decrease in enzyme activity. The lower activity towards guar gum was supporting the hypothesis that the mannanase activity gets affected by the degree and number of branched α-galactose residues on the main chain of heteromannans[3,6,24]. It poorly hydrolyzed

Table 2. Substrate specificity of β-mannanase of *P. thiaminolyticus*

Polysaccharides	Relative enzyme activity (%)
Locust bean gum	100 ± 4.28
Konjak glucomannan	98.25 ± 5.22
Guar gum	35.5 ± 0.50
Mannan (INM)	10 ± 2.28
Starch	72.25 ± 4.19
Xylan	ND
Pectin	ND
CMC	ND
Chitin	ND

ND-Not detected.

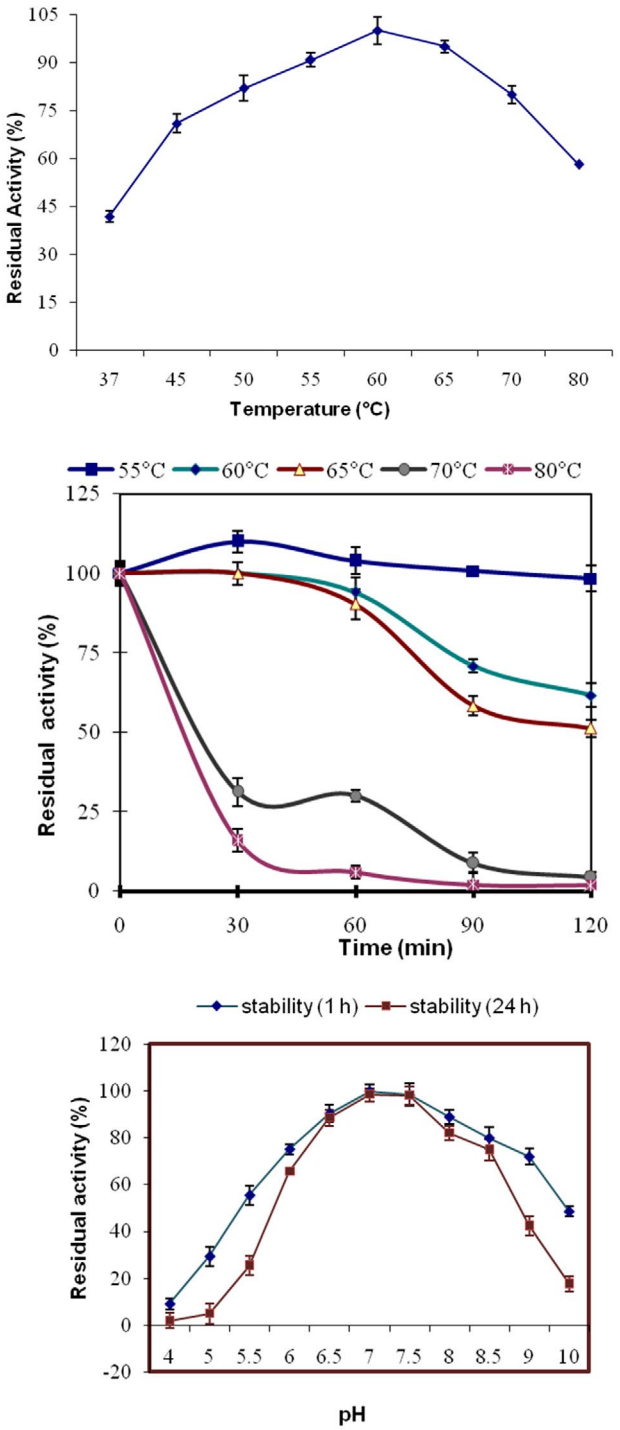

Fig. 3. (a) Effect of temperature on the activity of purified β-mannanase from P. thiaminolyticus. (b) Effect of temperature on thermal denaturation of β-mannanase After preincubation at 55°C (Squares), 60°C (filled diamonds), 65°C (filled triangles), 70°C (filled ovals) and 80°C (colored cross) for various lengths of time followed by enzyme assay at 60°C. 3 (c). Effect of pH on the stability of purified β-mannanase from P. thiaminolyticus. The pH stability was determined by measuring the residual activity after incubation at various pH values at 55°C for 1 h and 24 h.

unbranched β-1, 4—mannan (INM) homopolymer. This result suggested that purified mannanase is able to hydrolyze substituted mannans more effectively as substrates. Purified enzyme hydrolyzed the starch, but it was not active on either CM-cellulose, xylan or pectin, signifying that it could not cut the β-1, 4-cellulosic linkages and did not have xylanolytic and pectinolytic activity.

Enzyme kinetics was studied with LBG as substrate. The kinetic parameters obtained correspond to a V_{max} of 1111.11 U/mg and a K_m of 5 mg/ml for the β-mannanase (Fig. 4). Kinetic constant (K_m) and the maximal reaction velocity (V_{max}) values of β-mannanases, are described for various bacteria. For *B. subtilis* SA–22 V_{max}- 188.68 µmol min−1 ml−1 was reported for Locust bean Gum[25], Whereas, in *Bacillus subtilis* evident V_{max} values of the mannanase for locust bean gum, guar gum and konjac powder were 970.3 ± 10.3, 556.4 ± 15.2 and 435.3 ± 29.1 µmol min^{-1} ml^{-1} respectively[17]. kinetic comparisons remained difficult in context to β-mannanase as pattern and extent of branching of polymeric chains of mannose vary with different galactomannans isolated from different sources. The lack of standardization in the substrates utilized, their method of preparation and variations in the temperature and pH conditions during the assay make it difficult to compare the kinetic constants of mannanases from different microorganisms.

Effect of stabilizers/ organic solvents on mannanase activity and stability

The significant effect of different organic solvents ethanol, methanol, propanol, DMSO, glycerol, propanone, ethylene glycol, polyethylene glycol and iso-amyl alcohol on enzyme activity and stability was determined at 30% (v/v) concentration. Residual β–mannanase activity revealed that Glycerol, DMSO, ethylene glycol, polyethylene glycol (PEG) had a stimulatory effect on the mannanase activity. Ethanol, methanol and

Table 3. Effect of stabilizers/ organic solvents on mannanase activity and stability

Organic solvents 30% (v/v)	Residual activity after 1 h	Residual activity after 24 h
Control	100 ± 1.42	100 ± 3.25
Ethanol	31.2 ± 2.15	11.77 ± 1.54
Methanol	80.92 ± 3.44	15.77 ± 1.22
Propanol	21.38 ± 0.42	12.01 ± 4.15
DMSO	121.8 ± 1.50	111.4 ± 1.70
Glycerol	111.15 ± 1.00	113.22 ± 3.16
Propanone	39.34 ± 4.42	14.07 ± 3.42
Ethylene glycol	112.76 ± 1.85	127.66 ± 2.65
Poly ethylene glycol	124.19 ± 1.85	126.09 ± 3.18
Iso-amyl alcohol	114.3 ± 2.75	72.25 ± 4.45

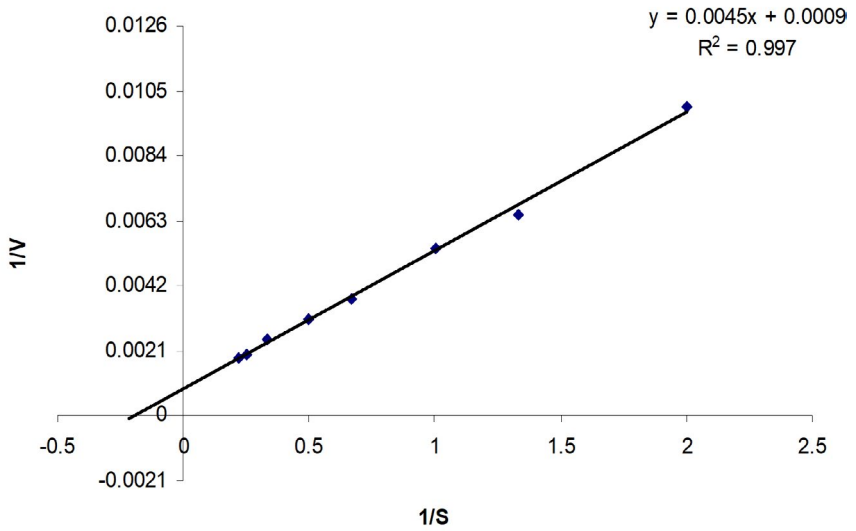

Fig. 4. Reciprocal velocity plot (Lineweaver-Burk's plot) of purified β-mannanase activity as a function of locust bean gum concentration.

propanol caused almost 70%, 20%, 80% inhibition respectively after 1 h and near complete inhibition after 24 h. Propanone resulted in almost 60% inhibition after 1 h and 86% inhibition was observed after 24 h. Iso-amyl alcohol had a stimulating effect on mannanase activity initially, but prolonged incubation resulted in 28% inhibitory effect (Table 3). β-mannanase responded differentially to water-miscible/immiscible solvents[26], but polar water miscible solvents were found to be more destabilizing for it[27]. As glycerols and polyols have stabilizing effect on the purified enzyme, allowing the use of organic solvents to prevent the enzyme loss during storage. Polyols act by stabilizing hydrophobic interactions that help to maintain native structure and preserve the hydration shell around the protein molecule.

Suitability of β-mannanase for detergent application

E numbers are codes for permitted food additives classified by European Food Safety Authority. Heteromannans Locust bean gum and guar gum are two of the most commonly used thickeners, designated by the E-numbers E410 and E412 respectively[28]. Guar gum and locust ban gum are versatile food additive present in instant noodles, pasta, Ice-cream, BBQ Sauce, Mayonnaise, salad dressing, thus generating tough and difficult to remove stains.

Various Food and personal care products which soil the clothes contain these additives, owing to their high molecular weight; they exhibit a strong adherence to fabric. These colorless gums though appear to be removed from clothes in the wash, but as they are strongly adhered to the fabric, particulate arising from wear of the garment or coming from a second wash can bind to garment and reappear as stains. Treatment with β-mannanase can reduce this reappearing stain phenomenon, can recover paleness and can prevent binding of certain soils to the cellulosic material. Enzymes being highly specific in action, nonspecific hydrolysis of mannan are not carried out by other glycoside hydrolases- amylases and cellulases usually present in detergents as they do not recognize the structure of mannan and cannot therefore remove gum based stains from fabric[29,30].

For an enzyme to be used as detergent additive, it should possess the following characteristics to make it useful as detergent enzyme: Stability - in Broad pH range, at relatively high temperatures (35-70°C) and in the presence of certain surfactants and detergent additives with specific substrate specificity.

Effect of surfactants on enzyme activity and stability

β-mannanase possessing activity in the broad pH range was recognized as potential detergent additive and part of stain-removing formulations. Taking this into consideration, the effect of various ionic and non-ionic surfactants on mannanase activity was investigated. The β-mannanase retained significant activity in all the nonionic surfactants viz. Triton-X-100 (TX-100), Polysorbate-20 (PS-20) and Polysorbate-40 (PS-40). These non-ionics possess no electrical charge on hydrophilic ends, which makes them averse to interact with Ca^{2+} and Mg^{2+} ions in hard

Table 4. Effect of various detergent additives on enzyme stability of purified β-mannanase from *P. thiaminolyticus*

Detergent additive	Class	Residual activity (%)
Sorbitol	Builder	96.9 ± 2.50
Mannitol	Builder	91.48 ± 1.76
Zeolite	Builder	33.67 ± 2.9
Citric acid	Builder	10.52 ± 1.25
H2O2	Bleaching agent	92.2 ± 0.25
Sodium perborate	Bleaching agent	28.93 ± 2.67
PVP	Dye tranfer inhibitor	95.2 ± 3.37
CM-cellulose	Anti redeposition agent	90.7 ± 4.67
PEG	Optical brightener	124.19 ± 2.82
Sodium sulfate	Bulking agent	89.45± 3.52

The purified enzyme was incubated at 60°C for 1 h in the presence of different detergent additives and the residual activity was determined

water making them hard water resistant. They are excellent degreasers extensively used in laundry detergents, and dishwashing liquids[31]. Addition of 0.4 to 1.0% (v/v) of the cationic surfactants cetyl-trimetylammonium bromide (CTAB) led to total inhibition of mannanase activity. Fig. 5 (a) shows the effect of PS-20, PS-40, TX 100, CTAB and SDS on the activity of mannanase. The effect of surfactants on the enzyme denaturation was examined further by exposing the enzyme to different concentrations of surfactants at different time intervals Fig. 5 (b). Different SDS concentration (1, 0.1 and 0.01%) led to about 80% of enzyme activity loss in 15, 30 and 60 min respectively. These findings agreed well with the studies reported earlier. Several enzymes interact with SDS to form enzyme-SDS

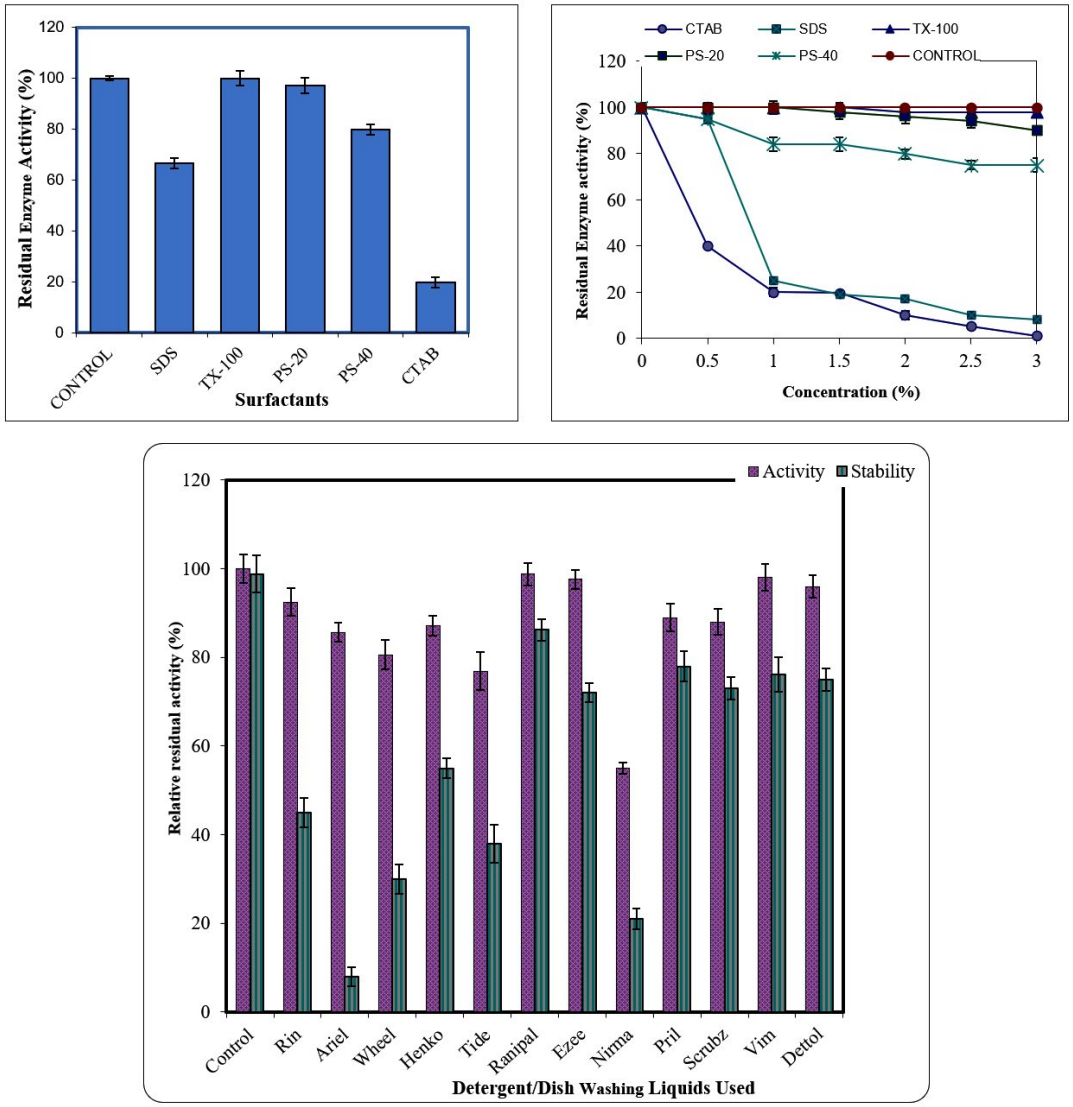

Fig. 5. (a) Effect of various surfactants on enzyme activity of purified β-mannanase from *P. thiaminolyticus.* (b) Effect of various surfactants on enzyme stability of purified β-mannanase from *P. thiaminolyticus*. The purified enzyme was incubated at 60°C for 1 h in the presence of different concentration of surfactants and the residual activity was determined. (c) Effect of various detergents/dish washing liquids on enzyme activity and stability of purified β-mannanase from *P. thiaminolyticus.* The purified enzyme was incubated at 60°C for 45 min in the presence of detergents/dishwashing liquids and enzyme stability (grey bars) was determined.

complexes. Generally at low concentration, SDS bind the outer surface of the enzyme, perhaps higher SDS concentration induced a larger conformational change leading to unfolding and enzyme inactivation[31,32]. Thus, Inhibitory effect of SDS depends upon its concentration and incubation time for denaturation of mannanase.

Effect of detergent additives

The β-mannanase showed significant stability with various detergent additives i.e. builders (sorbitol, mannitol, zeolite and citric acid) and bleaching agents (H_2O_2, sodium perborate). It also displayed excellent stability along with occurrence of other detergent components like optical brighteners (Polyethylene glycol), anti-redeposition agents and soil suspension agents (CM-cellulose), dye tranfer inhibiting agents (Polyvinyl pyrollidone) and bulking agent (Sodium sulfate). (Table 4).

Compatibility with laundry detergents

The *P. thiaminolyticus* β-mannanase showed moderate activity and compatibility along with commercial detergents (Rin, Ariel, Wheel, Tide, Henko, Nirma, Ranipal and Ezee). The mannanase maintained more than 50% activity after 15 min incubation at 60°C with most of the detergents tested but retained 100% activity in the presence of Ranipal and Ezee Fig. 5 (c). However on increasing exposure time to detergents β-mannanase retained less than 10% activity (data not shown). Thus, to overcome the effect of variable pH of different washing powders on enzyme stability when the effect of all washing powders (pre adjusted at pH 7.0) was checked, β-mannanase retained 55.2%, 46.7% and 38.5% activity in Henko, Rin and Tide respectively, but less than 30% in Nirma

Fig. 6. (a) Images of chocolate ice cream spot on cotton cloth. (b) Images of chocolate ice cream spot taken after washing in TX-100 with detergent additives without mannanase and after additional particulate soil treatment. (c) Images of chocolate ice cream spot taken after washing in TX-100 with detergent additives with mannanase and after additional particulate soil treatment.

Fig. 7. Images of anti-redeposition effect of mannanase with tennis court clay on cotton cloth (a) without mannanase (b) with mannanase.

and Wheel. It was observed that Ariel was very destabilizing for this enzyme, whereas enzyme was 90% and 72% stable in Ranipal and Ezee respectively (Fig. 5c). This inhibition of the enzyme activity by different detergents could be due to their ingredients and not by their high alkaline nature[33]. Many detergent additive enzymes possess even lesser activity than this[34]. Srivastava et al., (2014) reported an endo-mannanase from *Bacillus* sp. CFR1601 showing compatibility with various detergents. Enzyme retained about 89-100 % activity in detergents but at a temperature of 37°C[6]. In comparison β-mannanase from *P. thiaminolyticus* was significantly stable even at a temperature of 60°C in commercial detergents. As high temperature washing is preferred aiding to better cleaning.

Compatibility with dish washing liquids

Various brands of liquid cleansers, dishwashing liquids readily available in local market were evaluated for pH. Real-life situation was simulated by preparing a dilution of each cleanser with tap water and then change in pH was noted. Most of it was showing wide ranging pH in the range 4-8 and compatibility with normal skin pH. Without neutralizing the pH of Dishwashing liquids, β-mannanase retained about 90 % activity in Vim, Pril, Scrubz and Dettol Kitchen Dish washing Liquids respectively, and more than 70 % even after exposure time of 45 min at 60°C in these dish washing liquids (Fig. 5c). So this enzyme could have a potent application as an additive in dishwashing liquids as heteromannans are a component of many BBQ sauces, ketchups and instant foods as thickening agent generating tough and sticky stains. β-mannanase degraded heteromannan reduced the viscosity of residual food in cooking vessels and dishes thus easing out cleaning of tough stains thereby facilitating wash out of sticky stains (image not shown).

Cleaning performance test

In order to study the potential of mannanase as detergent additive for laundry, white cotton swatches (8 X 8 cm) were stained with chocolate ice cream (Fig. 6 a-c) and salad dressing (image not shown). Stained spots were allowed to dry at room temperature. To check cleaning performance of mannanase, before washing, all the stained swatches were dipped/ immersed in the wash solution (flasks a-f) at 60°C for 30 min. the test swatches were rinsed with water twice for 5 min and then dried for determining the washing performance. Among all the tested detergents/surfactants, TX-100 and PS 20 were most suitable surfactants for enzyme action. Cleaning performance study demonstrated that flasks (c-f) were containing effective wash solutions.

A lot better stain removal effect was easily noticeable when mannanase was added to TX-100 and commercial laundry detergent (Fig. 6c). Enzyme quickly cut the mannan based gums thus hydrolyzing the stain which is easily removable along with wash water. After stain hydrolysis, Mannan gum dirt/soil residues in the wash liquor can act like dirt attracters. Because of their sticking tendency to cellulose fibers can cause redeposition or back stain on other clean fabrics in the wash liquor.

Anti-soil redeposition effect of mannanase was studied by treating cotton swatch with tennis court clay and LBG, it was observed that in the absence of mannanase cotton swatch emerged heavily dirty with tennis court clay (Fig. 7a). When β-mannanase is present, it cut down gum based polymers quickly suspending and emulsifying into smaller more water soluble oligosaccharides, which do not stick to the fabric and are easily swirled out of the wash and maintaining the whiteness of the fabric and inhibiting redeposition and back stain on otherwise clean cloths (Fig. 7 b).

Based on the stability of purified enzyme with different surfactants and commercial detergents, it was observed that the mannanase derived from *P. thiaminolyticus* may get damaged by commercial detergents, perhaps induced by conformational change leading to unfolding and enzyme inactivation[31,32]. Since composition of the commercial detergents was not very clear because none of the label contained the information about the type of surfactants (anionic and nonionic) present, and the type of enzymes used in the composition. Hence it can be concluded that if a detergent composition was specially developed while keeping in mind the present enzyme, better cleaning efficiency can be obtained. For the reason of its extensive thermal stability and compatibility with surfactant and detergent additives, this mannanase could have specialized industrial application as an additive or specifically a rinse aid

composition for laundry[30]. A specialized cleaning rinse aid composition comprising a detergent ingredient selected from nonionic surfactant; a builder (sorbitol, mannitol, zeolite) and a bleach system with optical brighteners, will fulfill all the requirements mentioned earlier for its application in laundry detergent formulations.

As mannanase in the present study is capable of effectively degrading or hydrolyzing any soiling or spots containing galactomannan, glucomannan and, thus is capable of cleaning dishes comprising such soiling or spots, Moreover, β- mannanase from *P. thiaminolyticus* was showing wide-ranging temperature stability in the range of 35-60°C for more than 1 h which is the normal washing time even in automatic dish washers.

The enzyme used in this study was effective at low level, compatible with various detergent components, active at wide range of temperatures and capable of removing stained spots. The surfactant class, enzyme characteristics, and ratio of amalgamating ingredients need to be considered when mixing enzyme with detergent for achieving a better cleaning performance for a particular application[33].

In conclusion it can be said that this purified mannanase has many desirable characteristics, viz., high thermal activity, stability in broad pH conditions, high hydrolytic activity towards heteromannans LBG and KGM, no cellulase activity and high specific activity combined with the characteristic of certain metal ion and protease resistance, meet the criteria for this β-mannanase to be added as an additive for both dishwashing and laundry detergents.

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DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies with human participants or animals performed by any of the authors.

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