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RESEARCH ARTICLE

Purification and Characterization of A β-Mannanase from *Lactobacillus plantarum* (ATCC® 14917TM)

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HIGHLIGHTS

> Mannanase was purified alkaline thermo *Lactobacillus plantarum* (ATCC® 14917TM).
> Mannanase enzyme was purified using ammonium sulfate precipitation and DEAE-Cellulose ion exchange chromatography and Sephacryl S200.
> All the metal ions were increased the enzyme activity from 100 to 344 %.

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ABSTRACT

Mannanase alkaline was purified from thermo *Lactobacillus plantarum* (ATCC® 14917TM) using ammonium sulfate precipitation and DEAE- cellulose ion exchange chromatography and Sephacryl S200 Gel filtration chromatography techniques, in 36% yield and 111 -fold. Gel filtration chromatography and SDS-PAGE electrophoresis were executed pure enzyme systems 35 kDa and 55 kDa. Enzyme subunits were determined as two subunits. Optimum pH 10 and 40 °C was determeined at the optimum temperature. Enzyme activities were retained fairly between 30-80 oC. It was observed that purified mannanase in a wide range was mostly stable at elevated temperatures. In addition, the effects of some metal ions such as Ca2+, Mn2+, Co2+, Zn2+, Cu2+, Fe2+ and Ni2+ on mannanase enzymes activity was assayed and all the metal ions were increased the enzyme activity from 100 to 344 %.

Contents

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1. Introduction

β-Mannanases are extracellular enzymes hydrolyzing the 1,4-n beta-D-mannosidic linkages in mannans, galactomannans, glucomannans and galactoglucomannans. Mannans and widely heteromannans hard and soft woods, legumes and beans as part of the hemicellulose fraction is distributed in nature. Hemicelluloses are copolymers of both hexose and pentose sugars. The branched structure allows hemicellulose to exist in an amorphous form that is more susceptible to hydrolysis.

The demands for microbial enzymes for industrial application have increased in the search of Microorganisms with potential and adoption of biotechnological methods of enhancing enzyme production [1]. Biotechnology research into microbial enzymes has been driven by the need to isolate and identify organisms which are either hyper-producer and/or sufficiently robust to with stand conditions of the intended application and/or are producers of novel extra cellular enzymes. Therefore, importance is placed on industrial enzymes of microbial origin with catalytic efficiency that is relatively stable under wide pH range, high temperature, and salts concentration with a view of producing industrial enzymes of desirable physicochemical properties [2].

In recent years, mannanases (1,4-*β*-D-mannanmanno hydrolase, EC 3.2.1.78) have gained increasing attention because of their various biotechnological applications in the food, feed, coffee extraction, oil drilling, detergent, as well as pulp and paper industries [3, 4]. They can also be used in the production of mannooligosaccharides, which were reported to be excellent prebiotics stimulating growth of beneficial intestinal microorganisms [5, 6]. Many microorganisms have been reported as mannanase producers. Microbial species known to actively produce mannanases include *Bacillus subtilis* [7, 8], *Aeromonas hydrophila* [9], *Enterococcus casseliflavus* [10], *Pseudomonas* sp. [11], *Vibrio* sp. [12], *Streptomyces* sp. [13], fungi [14], and yeast [15]. Mannanases are also distributed in higher plants [16] and animals [11, 17]. Although it is presumed that the hemicelluloses are degraded both aerobically and anaerobically in natural environments, there are no reports available concerning mannanase production or β-1,4- mannan-degradation by anaerobic bacteria in natural environments.

In this experiment; under optimum conditions of temperature, pH and metal resistance, *L. plantarum* which produce mannanase enzyme was obtained purified and characterized using ion exchange and gel filtration chromatographic techniques.

2. Materials and methods

2.1. Characterization of Lactic acid Bacteria (LAB)

Lactic acid bacteria used in the study, strain *L. plantarum* (ATCC® 14917™), has been obtained from Sigma-Aldrich. Our bacteria have been transferred to MRS broth. *L. plantarum* bacteria were incubated in liquid medium at pH 5-7, at 30-35 °C range for 48 hours. After 7 days of incubation, mannanase enzyme activity was determined.

2.2. Purification of mannanase from *L. plantarum*

Subsequent to the filtering process, the crude enzyme extract was centrifuged at 5000 rpm for 15 min and the enzyme homogenate ammonium sulfate was precipitated at 0-20% 20-40% 40-60% 60-80% and 80-100% intervals, respectively. Following the filtration, raw enzyme extract was centrifuged at 5000 rpm for 15 min.

The obtained supernatant was saturated up to 40-80%, using ammonium sulphate. Thereafter, the precipitate was dissolved in 20 mM sodium citrate (pH: 6.0), and it was dialyzed against the same buffer.

2.2.1. Anion exchange chromatography

Suspension dialyzed ammonium sulfate, DEAE-cellulose ion exchange column (2.5 x 30 cm) was performed after it was previously equilibrated with 20 mM sodium citrate (pH: 6.0). Column was washed with the same buffer. Afterwards, the proteins attached to the column were eluted by applying NaCl gradient from 0 up to 1 M. The fractions were collected as 3 mL, with a 3 mL/min flow rate. Protein elution absorbance was measured at 280 nm as spectrophotometrically. Hyrolysis activities of locust bean gum were determined for all enzyme fractions. Active fractions were combined and it was allowed to stand at 4 °C.

2.2.2. Gel filtration

By using an Amicon membrane concentrator with a 10 kDa cut off, the active fractions which were obtained from anion exchange column were combined, dialyzed, desalted and concentrated, respectively. The aforementioned method, obtained by enzyme solution, previously equilibrated with 20 mM phosphate buffer (pH 5.0) to the Sephacryl S-200 column (120 x 1 cm), then the enzyme was eluted using 0.5 M of same NaCl-containing buffer. After all the fractions had been analyzed as described above the active fractions were combined, concentrated, they were allowed to stand at 4 °C for later use.

2.3. Protein concentration

By the method of Bradford [18], the protein concentration, as mentioned previously, are determined spectrophotometrically. In this method, bovine serum albumin (BSA) was used as a standard.

2.4. Determination of mannanase activity

The mannanase activity was principally determined by using a locust bean gum as a substrate [19]. The mixture of the reaction was formed by adding a 0.1 mL enzyme solution onto the locust bean gum substrate dissolved with 0.9 ml of 20 mM sodium citrate buffer (pH: 6.0).

After this process, it was incubated at 50 °C, in which the mannanase activity was determined through monitoring the absorbance at 540 nm by adding in the increased an average of DNSA (3,5-dinitrosalisilic acid) [20].

Mannanase activity was determined by taking the measurements at 540 nm using locust bean gum as substrate. Km and Vmax values were determined. Blind sample was prepared using distilled water [19].

2.5. SDS polyacrylamide gel electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method was identified by Laemmli [21], 10-30% discontinued, was realized.

20 µg protein sample, each being disposed into a gel casting apparatus (Bio-Rad) was applied to buffer (0.25 M Tris, 1.92 M glycine, 1% SDS, pH the electrophoresis process: 8.3) at 4 °C. Gel were stained in a 50% methanol, 10% acetic acid and 40% distilled water mixture for 1.5 hours, until it was clear. Afterwards, electrophoretic pattern was photographed.

2.6. Molecular weight determination by gel filtration

Sephadex G100 column (3.0 x 70 cm) was used for determination of the molecular weight of mannanase enzyme. Column was balanced using 0.05 M Na_2HPO_4, 1 mM dithioerythretol pH 7.0 buffer.

Standard protein solution (bovine serum albumin, 66 kDa; egg ovalbumin, 45 kDa; pepsin, 34 kDa; trypsinogen, 24 kDa; ß-lactoglobulin and lysozyme, 14 kDa) was applied to the column and eluted from the column with the same buffer. Afterwards, the pure mannanase enzyme was applied to the column and eluted under same conditions. The flow-rate through the column was 20 mL/h. The elution volume was compared with those of standard proteins [22].

2.7. Studies on certain *in vitro* chemicals and metal ions

Effect of different concentrations of inhibitors (Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+} and Ni $^{2+)}$ on mannanase enzyme activity was investigated. All the metal ion concentrations were tested for each of the three runs. Mannanase inhibitor/activator activity was measured at different concentrations.

2.8. Statistical analysis

For all testing samples, mannanase activity was determined at three different runs. Data were expressed as mean ± standard error. Using SPSS 20.0 software (SPSS Inc, Chicago, IL., USA), statistical analysis had been performed. Significant differences were determined as 95% confidence intervals ($p < 0.05$) using Tukey's test.

3. Results and Discussion

3.1. Purification and characterization of mannanase from *L. plantarum*

The mannanase enzyme manufactured extracellularly from the bacteria called *L. plantarum*, was refined in 3 steps as ammonium sulfate precipitation, DEAE-cellulose and Sephacryl S200 gel filtration chromatography. All the purification steps of the mannanase enzyme are shown in Table 1.

In the first stage, mannanase enzyme was collapsed the ammonium sulfate saturation between the range 0-90%. Highest percentage of precipitation of mannanase enzyme was obtained at 60-80% range. 33 purification-fold and 74.5% yield was achieved in this first purification step (Table 1).

In the second step, sample was applied to the DEAE-cellulose ion exchange column, enzyme rose to 31.5% and a yield 69 times greater purified-fold.

In the last step, obtained fractions from ion exchange column were concentrated, then it was applied to a Sephacryl S 200 column. A single peak was obtained from this column, and 110.6 purification-fold and 36% yield. 22 EU / µg protein, specific activity (Table 1) was calculated from results.

Table 1 The purification process of purified β -mannanase enzyme from *L. plantarum*

Enzyme Fraction		Crude extract	$(NH_4)_2SO_4$ (40-80%)	DEAE-Cellulose	Sephacryl S200
Volume (mL)		50	48	20	20
Activity (EU/mL)		41.6±1.3	31.5±1.3	15.2±0.32	7.78±2.5
Total Activity	EU	$2.08x10^3$	$1.51 x10^3$	$3.04 x10^2$	$1.56 x10^2$
	%	100	72.7	20.1	51.2
Protein (µg protein/mL)		152.8±1.8	3.88±0.38	0.92±0.11	0. 18±0.4
Specific Activity (EU/µg protein)		0.272	8.12	16.52	43.2
Purification Fold		-	29.85	60.74	158.8

Mannanases from bacteria elution profiles of anion exchange chromatography and gel filtration was purified by chromatography, *L. plantarum*, is presented in Figure 1 and Figure 2.

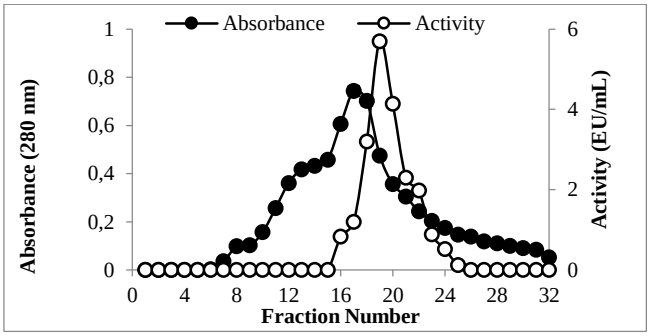

Figure 1 Purification of β-mannanase enzyme from *L. plantarum* by ion exchange chromatography using DEAE-Cellulose.

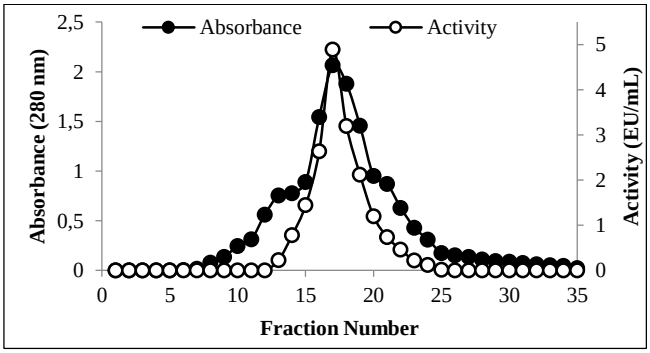

Figure 2 Elution profile of protein and β -mannanase of *L. plantarum* activity on Sephacryl S200.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) results of the purified mannanase enzyme were observed to have consisted of two subunits as 35 kDa and 55 kDa (Figure 3). To find the molecular weights of the active form of the enzyme, the Sephadex G-100 gel filtration chromatography was applied and the graph was drawn as *log* MA-Kav.

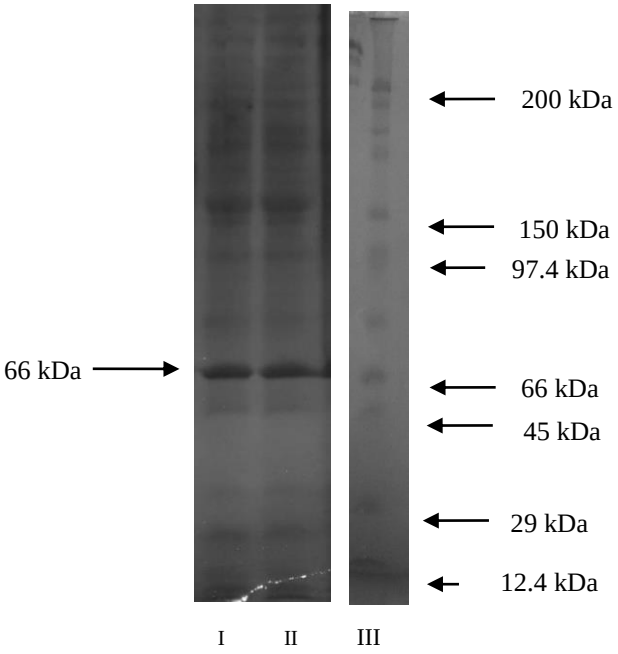

Figure 3 SDS-PAGE electrophoretic pattern of peroxidase [standart protein (β-Amylase, sweet potato, 200 kDa; alcohol dehydrogenase, yeast, 150 kDa bovine serum albumin, 97.4 kDa; rabbit muscle phosphorylase A, 66 kDa; egg ovalbumin, 45 kDa; pepsin, 29 kDa; carbonic anhydrase); cytochrome c, horse heart 12.4 kDa (I); purified laccase enzyme from rocket peroxidase (II)].

In this study, the amplified beta-mannanase gene was placed from *Aspergillus aculeatus* to *Aspergillus oryzae* and the molecular weight of the resultant recombinant enzyme was found to be 45 kDa [23]. Also, molecular weight of the mannanase enzyme of *Aspergillus niger* was obtained as 40 kDa [24].

Activity measurements were carried out using locust bean gum as a substrate, and the results were also calculated. Results were displayed using Activity - pH graph in Figure 4. The optimal pH values of the purified mannanase enzyme from *L. plantarum* (ATCC® 14917™), was determined to be 5.0 for the substrate locust bean gum.

The mannanase enzyme from *L. plantarum* was produced, purified and the pH optimum was found in range of pH 2 to 9. From pH 2 to pH 5, acetate buffer, from pH 6 to pH 8 phosphate buffer, and from pH 8 to pH 9 Tris / HCl buffer was used. Optimum pH was determined as pH 7.

Activity measurements, locust bean gum were used as a substrate, were calculated from the results obtained and were also plotted Activity - pH and the results were given in Figure 4. *P. plantarum* produced a purified mannanase enzyme and the optimal pH value was determined to be 5.0.

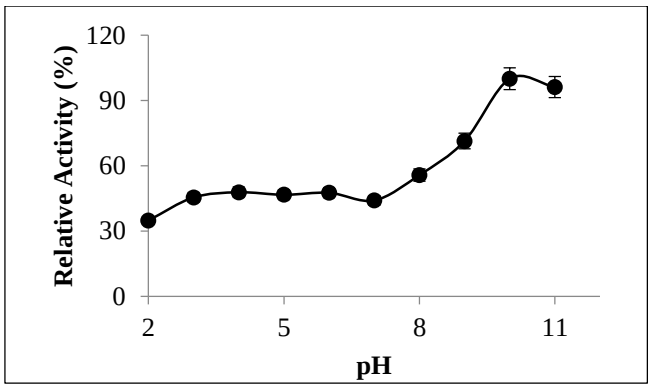

Figure 4 Effect of pH on the activity of the purified β -mannanase of *L. acidophilus*.

Enzymes and substrate were dissolved either in 10 mM buffers of various pH. Other conditions were as given for the standard assay method. Also, the pH stability was determined and related results were given in Figure 5.

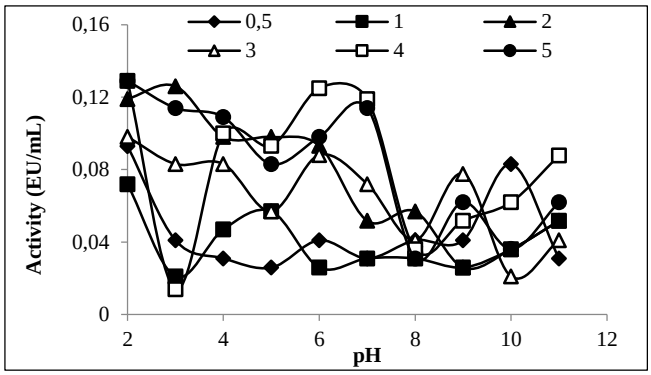

Figure 5 Effect of pH on the activity and stability of the purified purified β-mannanase of *L. plantarum*

3.2. Optimum temperature

To determine the optimum temperature of the purified mannanase enzyme in the range of 20-90 °C, an activity measurement was applied by keeping for 5 minutes at each temperature using the locust bean gum as a substrate. As seen in Figure 5, the mannanase enzyme was found to be quite active between 30-80 °C and exhibited the maximum activity at 60 °C (Figure 6). Similarly, the optimum pH results of the beta-mannanase enzymes were belong to *Aspergillus aculeatus* [23]. *Aspergillus niger's* mannanase optimal pH were determined to be 5.0, and the optimal temperature was also determined to be 60-70 °C [25].

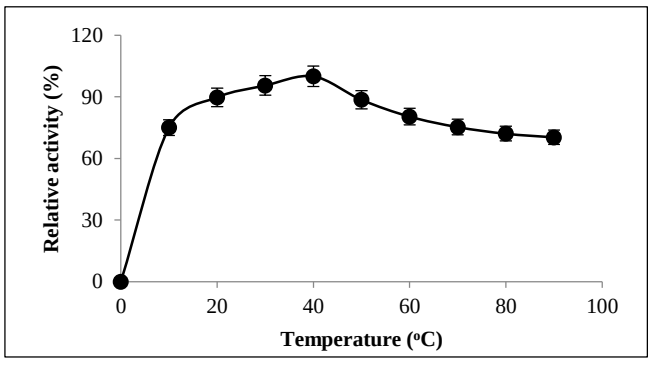

Figure 6 Effect of temperatures on the activity of purified β -mannanase of *L. plantarum*

Activity was determined at different temperatures and at pH 8.0 using the standard assay method. Also, the thermal stability was determined and related results were given in Figure 7.

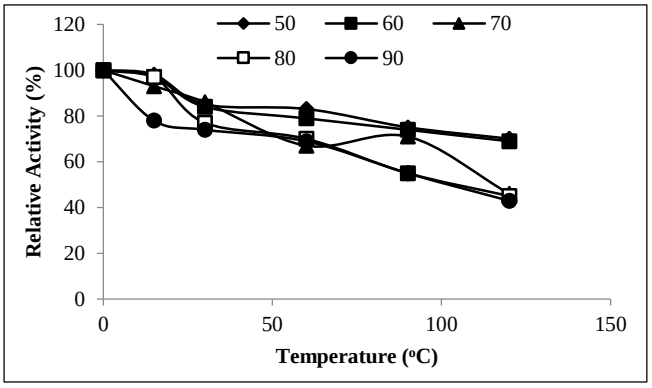

Figure 7 Effect of temperature on the activity and stability of purified β-mannanase of *L. plantarum*

3.3. The results of Vmax and Km values

Vmax and Km values of mannanase enzyme for 0.3 mM, locust bean gum was observed as 78.74 μmol/min.mg and Km 0.592 mM, respectively.

3.4. Effects of some metal ions on the activity of the mannanase enzyme purified from *L. plantarum*

The effects of the metal ions, such as Mn^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+} ve Ni^{2+} on the activity of the mannanase enzyme purified from *L. plantarum* (ATCC 14917) were investigated.

The inhibition/activation effects obtained as the result of the studies and the activity values versus concentration are shown in Table 2. As seen in Table 2, all the metal ions enhanced the activity of the purified mannanase enzyme from *L. palantarum*. However, the highest activation was done by Co^{2+} and Fe^{2+} metal ions, which enhanced the mannanase activity by 375.3 % and 372.8 %, respectively. From the results, it was observed that not all the metal ions inhibited the mannanase enzyme purified from *L. plantarum* (ATCC 14917) but that they even activated it, and due to such characteristic, it was considered to be quite convenient for industrial practices of this purified mannanase. It was determined that almost all of the metal ions increased the activity of purified mannanase enzyme from *L. palantarum* [26–29].

Table 2 The effect of some chemical compounds on β-mannanase activity.

Chemical Compound	Concentration (mM)	Relative Activity (%)	Concentration (mM)	Relative Activity (%)
None	-	100 ± 0.0	-	100 ± 0.0
Ca^{2+}	1	158.1 ± 0.62	5	136.8 ± 0.44
Mn^{2+}	1	213.4 ± 1.5	5	330.1 ± 1.7
Co^{2+}	1	326.5 ± 0.45	5	375.32 ± 2.1
Zn^{2+}	1	223.2 ± 1.3	5	180.7 ± 0.11
Cu^{2+}	1	210.0 ± 0.66	5	296.4 ± 0.81
Fe^{2+}	1	203.1 ± 1.7	5	372.8 ± 0.5
Ni^{2+}	1	220.0 ± 0.1	5	202.1 ± 0.8

4. Conclusion

It was concluded from the acquired findings that the catalytic activities of the mannanase enzyme purified from *L. plantarum* were rather high, and that they were resistant to the metal ions and were highly stable against different pHs and temperatures.

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