



## **Kinetic Properties of Purified $\beta$ -Mannanase from *Penicillium italicum***

**O. O. Olaniyi<sup>1\*</sup>, D. J. Arotupin<sup>1</sup>, B. J. Akinyele<sup>1</sup> and O. S. Bamidele<sup>2</sup>**

<sup>1</sup>Department of Microbiology, Federal University of Technology, P.M.B 704, Akure, Nigeria.

<sup>2</sup>Department of Biochemistry, Federal University of Technology, P.M.B 704, Akure, Nigeria.

### **Authors' contributions**

*This work was carried out in collaboration between authors. Author OOO designed the study, performed the statistical analysis, wrote the protocol, and wrote the first draft of the manuscript. Authors DJA, BJA and OSB managed the analyses of the study and the literature searches. The authors read and approved the first manuscript.*

**Original Research Article**

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### **ABSTRACT**

**Aim:** The study aimed at purification and characterization of  $\beta$ -mannanase from *Penicillium italicum*.

**Study Design:** The first experiment,  $\beta$ -mannanase from *Penicillium italicum* was produced in basal medium supplemented with Locust Bean Gum (LBG). The second described the purification of crude  $\beta$ -mannanase, while the third experiment dealt with characterization and kinetic studies of purified  $\beta$ -mannanase from *Penicillium italicum*.

**Place and Duration of Study:** Microbiology Research Laboratory, Federal University of Technology, Akure Nigeria between July and August 2012.

**Methodology:**  $\beta$ -mannanase from *Penicillium italicum* was produced in basal medium supplemented with LBG. The enzyme was purified by ammonium sulphate precipitation, ion exchange chromatography (DEAE-Sephadex A-50) and gel filtration (Sephadex G-150). The purified enzyme was characterized to determine its optimal conditions by standard assay procedures. The kinetic parameters of the purified enzyme were determined by Lineweaver-Bulk plot.

**Results:** Fractionation of ammonium sulphate precipitated  $\beta$ -mannanase from *Penicillium italicum* on sephadex A-50 produced one major activity peak. Further fractionation of partially purified enzyme from ion exchange on Sephadex G-150 yielded one activity peak. A pH of 5.0 was optimum for purified enzyme activity and relatively stable between 40 to 100 min of incubation at this pH. The optimum temperature was 70°C and 100%

\*Corresponding author: Email: microladit@gmail.com;

thermostable for 40 min after which a slight decline in activity was observed. The apparent  $K_m$  for the hydrolysis of LBG from Lineweaver-Bulk plot was approximately 0.26 mg/mL, while the  $V_{max}$  was 0.12  $\mu\text{mol}/\text{min}/\text{mL}$ . The incubation of salts and organic compounds at 10 mM and 40 mM caused inhibition of enzyme activity. At 20 mM, enzyme activity was enhanced by  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , SDS and  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , while others caused inhibition of enzyme activity. The incubation of enzyme with  $\text{CaCl}_2$  and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  at 60 mM enhanced enzyme activity, while others caused inhibition.

**Conclusion:** The result obtained from this study revealed that purified  $\beta$ -mannanase is active over a wide pH and temperature, and its stability implies that the enzyme will be useful during industrial processes where extreme conditions are required.

*Keywords: Kinetic properties,  $\beta$ -mannanase, purification, characterization, Penicillium italicum.*

## 1. 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  $\beta$ -1-4 linked mannose residues. Acetylated galactomannan, a principal component of hemicellulose, has a heterogenous backbone of  $\beta$ -1-4 linked mannose and glucose units. Mannanases (EC 3.2.1.78; 1,4- $\beta$ -D-mannan mannanohydrolases) occur widely in microorganisms such as fungi, yeasts, and bacteria as well as in germinating seeds of terrestrial plants [1,2,3,4,5].

Mannans function as carbohydrates storage in the bulbs and endosperm of some plants. Galactoglucomannans and glucomannans in softwoods and hardwoods are both branched heteropolysaccharides requiring several enzymes for their complete degradation [6].  $\beta$ -Mannanase (1,4- $\beta$ -D-mannan mannanohydrolase; mannan endo 1,4- $\beta$ -mannosidase; EC 32.1.78) is the enzyme that cleaves the  $\beta$ -1,4-mannosidic linkages of mannans, galactomannans, glucomannans, and galactoglucomannans [6, 7]. The  $\beta$ -mannanases have been grouped into two families, glycosyl hydrolase 5 (GH5) and glycosyl hydrolase 26 (GH26). The protein folding, catalytic mechanism and mechanism of glycosidic bond cleavage are conserved in both enzyme families [7].  $\beta$ -mannanase, required for the utilization of various  $\beta$ -mannans, occurs in certain endosperms such as copra and ivory palm nuts, in the beans of guar, locust, and coffee, and in the roots of konjak [6,8].

In recent years, mannanases have gained increasing attention because of their various biotechnological and industrial applications in the food, feed, coffee extraction, oil drilling, detergent, as well as pulp and paper industries [8,9,10,11]. They can be used in the production of manno oligosaccharides, which were reported to be excellent prebiotics stimulating growth of beneficial intestinal microorganisms [12]. Mannanases are useful in many fields including biobleaching of pulp and detergent industry [13], bioconversion of biomass wastes to fermentable sugars [14], and upgrading of animal feed stuff [15]. It can be used to reduce the viscosity of coffee extracts [16]. The coffee preparation using  $\beta$ -mannanases showed better volatile aroma, taste properties and visual appearance of the final drink [17]. In the present study, purification and characterization of  $\beta$ -mannanase from *P. italicum* was carried out.

## 2. MATERIALS AND METHODS

### 2.1 Fungi Isolate

*Penicillium italicum* isolated from agro-wastes previously confirmed positive for mannanase by plate assay in our previous work was used in this study [18]. The fungal isolate was identified in the Microbiology Research Laboratory, Federal University of Technology, Akure, Ondo State, Nigeria according to the method designed by [19] on the bases of cultural characters (colour, shape of colony, surface and reverse pigmentation and texture of the colony) as well as microscopic structure (septate or nonseptate hyphae, structure of hyphae and conidia). The fungal isolate was maintained on Locust Bean Gum (LBG) containing agar plates and sub-cultured at regular intervals. They were incubated at  $30\pm 2^\circ\text{C}$  until the entire plates were covered by active mycelium and stored at  $4^\circ\text{C}$  in refrigerator on agar slants.

### 2.2 Media Preparation and Enzyme Production

For the production of mannanase in submerged state fermentation, the isolate was grown at  $30^\circ\text{C}$  in 250 mL Erlenmeyer flask in Mandels and Weber's medium modified by [6]. This medium contained the following ingredients (g/L): LBG 10, Peptone 2, yeast extract 2,  $\text{NaNO}_3$  2,  $\text{K}_2\text{HPO}_4$  1,  $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$  0.5, KCl 0.5 and  $\text{FeSO}_4\cdot 7\text{H}_2\text{O}$  traces. Final pH was adjusted to 6.0 and then sterilized at  $121^\circ\text{C}$  for 15 min. After sterilization, flask was inoculated with two discs of 8 mm diameter of the organism from mannan-containing agar medium using sterile cup borer [6]. The flasks were incubated at  $30^\circ\text{C}$  for 5 days at static condition. Crude enzyme preparation was obtained by centrifugation at 5000 rpm for 10 min at  $4^\circ\text{C}$  using refrigerated ultracentrifuge (Centurion Scientific Limited). The supernatant was used as the crude extracellular enzyme source.

### 2.3 Enzyme Assays

Enzyme activity of supernatant collected at the end of centrifugation was determined using Spectrophotometer (Lab-Tech Digital Colorimeter). Mannanase activity was assayed in the reaction mixture composing of 0.5 mL of 50 mM potassium phosphate buffer pH 7.0 and 1% LBG with 0.5 mL of supernatant at  $45^\circ\text{C}$  for 60 min (modified method of [6]). Amount of reducing sugar released was determined by the dinitrosalicylic acid reagent (DNS) [20]. One unit of mannanase activity was defined as amount of enzyme producing 1 micromole of mannose per minute under the experimental conditions.

### 2.4 Protein Determination

The amount of protein liberated in the fermentation medium was evaluated according to the method designed by Lowry et al. [21] using Bovine Serum Albumin (BSA) as the standard.

### 2.5 $\beta$ -Mannanase Purification

The supernatant obtained after centrifugation was purified in three stages. First, 1000 mL supernatant was precipitated by adding 472 grams ammonium sulphate to achieve 70% ammonium sulphate concentration [22]. After centrifugation at 5000 rpm for 20 min at  $4^\circ\text{C}$ , the precipitate was then diluted in phosphate buffer 50 mM pH 7.0, and dialyzed in the same buffer. Secondly, the enzyme obtained after dialysis was purified by employing ion exchange column chromatography with DEAE Sephadex A-50 matrix ( $20\times 2.5$  cm, Pharmacia). The

fractions obtained were washed with 300 mL ion-free water, followed by 200 mL 0.01 M Tris-HCl buffer pH 8.0. The gel was eluted with concentration gradient of NaCl. The absorbance of each fraction (5 mL) collected was measured at 280 nm and the activity of  $\beta$ -mannanase of each fraction was determined. In the third stage, 2.5 mL of the concentrated enzyme from second stage purification process was loaded onto the column chromatography (2.5 cm in diameter and 30 cm high) which contained Sephadex G-150 (Pharmacia). Phosphate elution buffer at 50 mM pH 7.0 was applied with flow rate of 20 mL/h. A fraction of 5.0 mL was collected at interval of 30 min and the absorbance at 280nm was read using spectrophotometer (Jenway, 6305). Fractions with  $\beta$ -mannanase activity were pooled and concentrated in glycerol solution at 30°C.

## **2.6 Effect of pH on $\beta$ -Mannanase Activity**

Buffer (50 mM) of different pH ranging from 3.0 to 9.0 were prepared using different buffer system, Glycine-HCl, pH 3.0; acetate buffer, pH 4.0 and 5.0; phosphate buffer pH 6.0 and 7.0; Tris- HCl, pH 8.0 and 9.0. Each of this buffer solution was used to prepare 1% LBG solution used as substrate in assaying the enzyme. The assay was carried out according to standard assay procedure.

## **2.7 pH Stability of $\beta$ -Mannanase**

To observe the pH stability, purified enzyme was kept with different pH buffers ranging from 5.0 to 9.0 at room temperature for different time intervals up to 2 h. Thereafter enzyme activity was assayed.

## **2.8 Effect of Temperature on $\beta$ -Mannanase Activity and Stability**

$\beta$ -mannanase activity was assayed by incubating the enzyme reaction mixture at different temperatures, 10°C to 90°C for 3 min. Thermostability of the enzyme was also examined. Samples were incubated at various temperatures (50-80°C) for 60 min. The samples were withdrawn at 10 min interval and thereafter residual activities were evaluated under standard assay conditions.

## **2.9 Effect of Substrate Concentration and Determination of Kinetic Parameters**

The effect of substrate concentration [S] on the rate of enzyme action was studied using [S] values of 0.25 mg/mL to 10.00 mg/mL. The Lineweaver-Burke plot was made. Both the maximum velocity ( $V_{max}$ ) and Michaelis-Menten constant ( $K_m$ ) of the enzyme were calculated.

## **2.10 Effect of Metal Ion and Organic Compounds on Enzyme Activity**

A stock solution (10, 20, 40 and 60 mM) of different metals ions and organic compounds were prepared. Two milliliter of each salt solution was mixed with 2 mL of enzyme solution. The mixture was incubated for 5 min at room temperature. A 0.5 mL of the mixture was withdrawn and assay according to standard assay procedure.

### 3. RESULTS

#### 3.1 Fractionation on Sephadex A-50 and Sephadex G-150

The ammonium sulphate-dialysate fraction on sephadex A-50 tends to produce one major activity peak designated as A (Fig. 1). Further fractionation of the components of peak of *P. italicum* from ion exchange on Sephadex G-150 yielded one activity peak (B) as shown in Fig. 2.

#### 3.2 Summary of Purification Procedures

Three-step purification of  $\beta$ -mannanase was performed, and a 23.24-fold purification was achieved with 16.27 U/mg specific activity (Table 1). After 25% ammonium sulphate saturation of protein broth, the precipitates were dialyzed against 50 mM phosphate buffer pH 7.0 for up to 24 h at 4°C. Dialyzed protein was loaded onto a Sephadex A-50 column, which was eluted with the same buffer.  $\beta$ -mannanase activity was detected in fractions 9 to 20. These collected fractions were pooled and further purified on Sephadex G-150. The fractions collected from 24 to 38 were pooled and later used for the characterization studies.

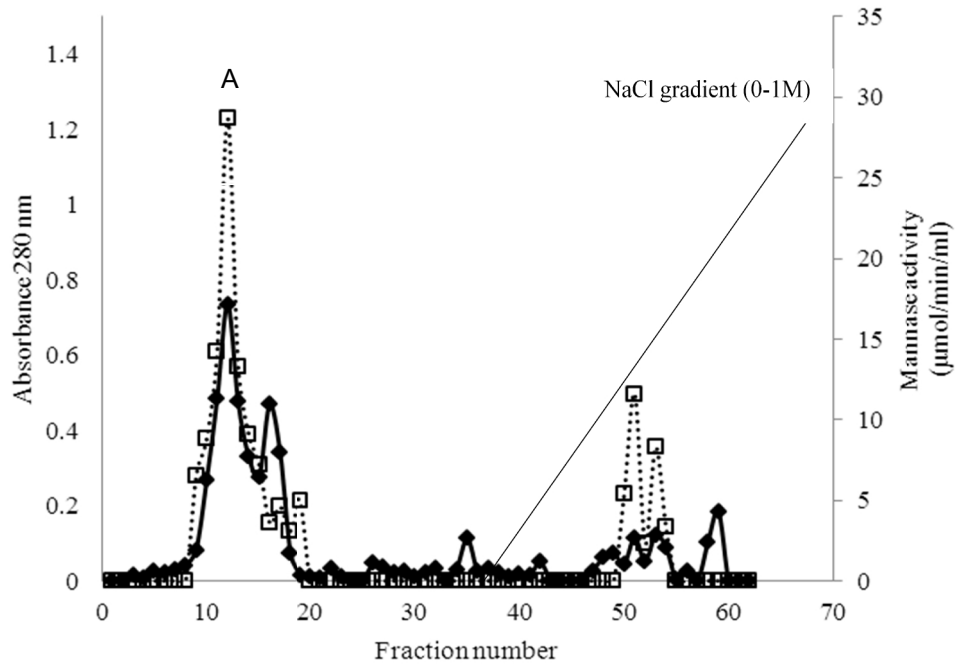
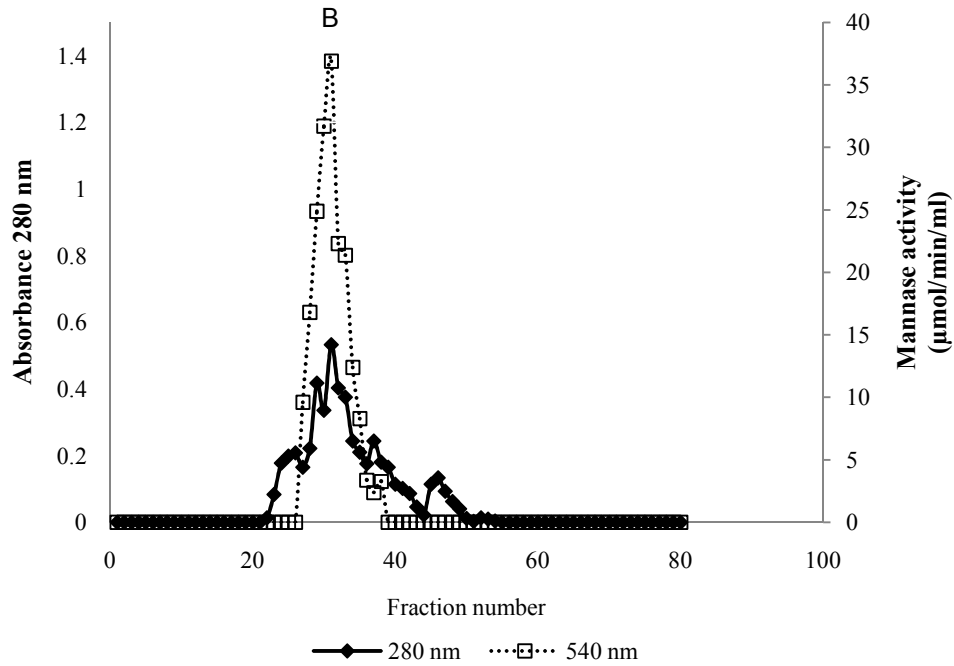


Fig. 1. Elution profile of crude mannanase on DEAE Sephadex A-50



**Fig. 2. Elution profile of partially purified  $\beta$ -mannanase on Sephadex G-150**

**Table 1. Purification table**

STEP	Vol	CP	EA	TP	TA	SA	Yield	Fold
Crude extract	25	7.5	5.25	187.5	131.25	0.7	100	1
Ammonium Sulphate precipitation	15	6.41	7.83	96.15	117.45	1.22	89.49	1.75
Ion exchange	8	2.32	12.59	18.56	100.72	5.43	76.74	7.75
Gel filtration	2.5	1.05	17.08	2.625	42.7	16.27	32.53	23.24

CP=Crude protein (mg/mL), SA=Specific activity ( $\mu\text{mol}/\text{min}/\text{mg}$ )  
 EA= Enzyme activity ( $\mu\text{mol}/\text{min}/\text{mL}$ ), TA=Total activity ( $\mu\text{mol}/\text{min}/\text{mL}$ )  
 TP=Total protein (mg)

### 3.3 Characterization of $\beta$ -Mannanase

#### 3.3.1 Effect of temperature and thermostability of $\beta$ -mannanase

The enzyme activity increased with increase in incubation temperature until an optimum percentage relative activity was achieved at 70°C (Fig. 3). The enzyme was thermostable for 40 min at 50°C after which a slight decline in activity was observed (Fig. 4).

#### 3.3.2 Effect of pH on activity and stability of $\beta$ -mannanase

The optimum percentage relative activity was attained at pH 5.0, whereas at pH 3.0 and 9.0 percentage relative activities reduced by 98% and 40% respectively when compared with the value obtained at pH 5.0 (Fig. 5). The stability of the enzyme was checked at different pH

ranging from 5.0 to 9.0 at room temperature for 2 h. The enzyme activity was relatively stable between 40 to 100 minutes of incubation at pH 5.0 (Fig. 6).

### 3.4 Effect of Substrate Concentration and Determination of Kinetic Parameters

The purified  $\beta$ -mannanase from *P. italicum* exhibited a  $K_m$  value of 0.26 mg/mL, when the substrate used LBG. Maximum velocity ( $V_{max}$ ) of this enzyme was 0.12  $\mu\text{mol}/\text{min}/\text{mL}$  (Fig. 7).

### 3.5 Effect of Salts and Organic Compounds on Enzyme Activity

The effect of different salts and organic compounds was evaluated on the activity of purified  $\beta$ -mannanase (Table 2). The incubation of salts and organic compounds at 10 mM caused inhibition of enzyme activity. At 20 mM, enzyme activity was enhanced by  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , SDS and  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , while others caused inhibition of enzyme activity. The incubation of enzyme with  $\text{CaCl}_2$  and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  at 60 mM enhanced its activity, while others caused inhibition.

## 4. DISCUSSION

Preliminary investigations revealed that *P. italicum* was capable of hydrolysing mannan [18]. Also, the presence of  $\beta$ -mannanase in the culture filtrate further corroborated the production of  $\beta$ -mannanase by this fungus. The hydrolysis of mannan-containing substrates had been reported [6,23,24]. The results obtained from this study is in conformity with the discovery of Madau and Setati [24], Sae-Lee [25] that mannanase is responsible for the considerably hydrolysis of mannan.

The results of this investigation showed that temperature at which the reaction mixture was incubated greatly affected enzyme activity. Similar optimum temperature (70°C) obtained for  $\beta$ -mannanase from *P. italicum* in this study was documented for mannanase from *Penicillium purpurogenum* [26] and *Geobacillus stearothermophilus* L-07 [22]. Different optimal temperatures had been reported for different microorganisms, optimum temperature of 50°C was reported for *Scopulariopsis candida* [27], 60 to 65°C was optimum for *Aspergillus fumigatus* [23]. The reasons for slight variation in temperature of afore mentioned studies had been well documented [27]. Inactivation due to heat has been associated with a two-step process. The reversible thermal unfolding of an enzyme as a result of increase in vibration and rotational motion of reacting molecules, which may lead to dissociation in case of multi-subunit enzyme [28]. The unfolding of the enzyme also exposes the reactive groups and hydrophobic areas, which can subsequently result in irreversible inactivation. Irreversible thermal inactivation could be due to protein aggregation as a result of exposure of the buried hydrophobic areas to solvent. Extremely high temperature could also lead to deamination of asparagines and glutamine residues, hydrolysis of the peptide bonds at aspartic acid residues, thiol disulphide interchange, and destruction of disulphide bonds and oxidation of amino acid side chains of protein molecule of the enzyme [29]. It has been reported that enzyme structure are disrupted and always a loss of activity at high temperature values above the optimum was observed [30]. Higher temperature optimum and temperature stability of  $\beta$ -mannanase from *P. italicum* makes it a more suitable catalyst for possible application in bio-bleaching and bio-pulping at higher temperatures.

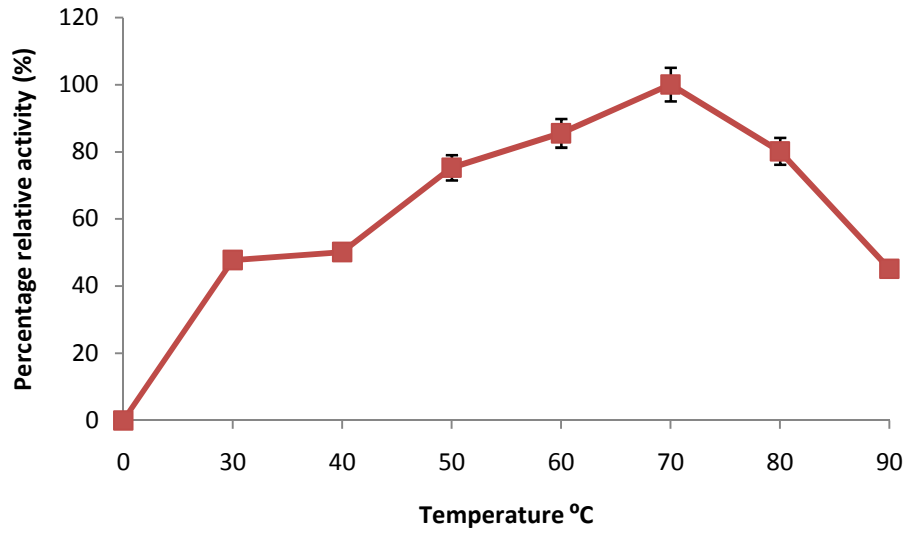


Fig. 3. Effect of temperature on  $\beta$ -mannanase activity

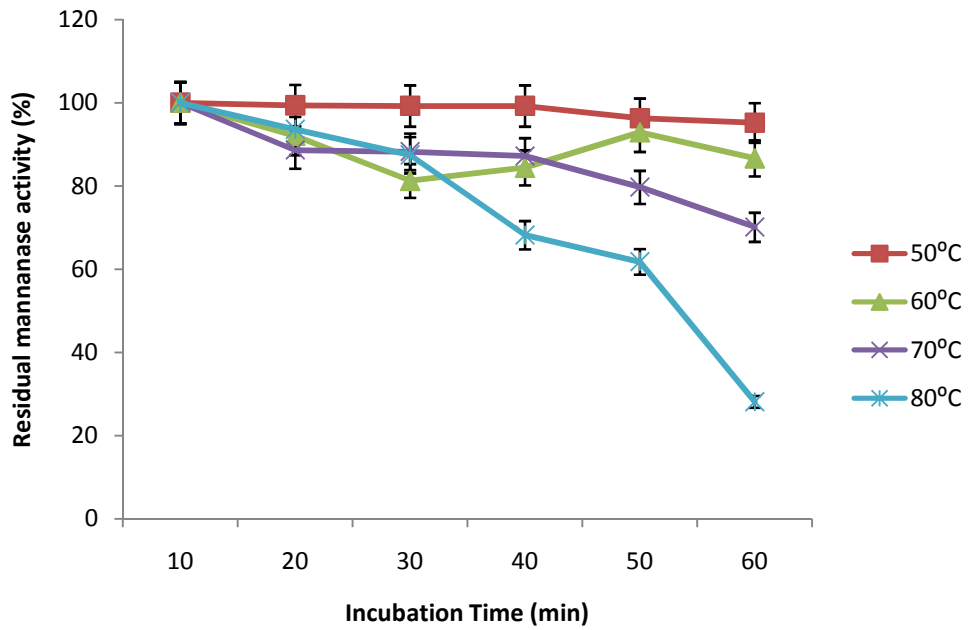


Fig. 4. Thermostability profile of  $\beta$ -mannanase



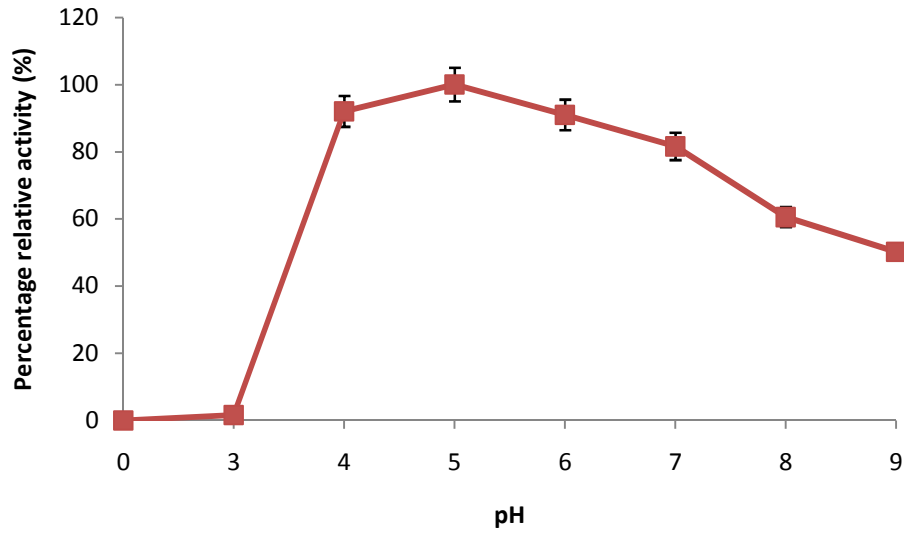


Fig. 5. Effect of pH on  $\beta$ -mannanase activity

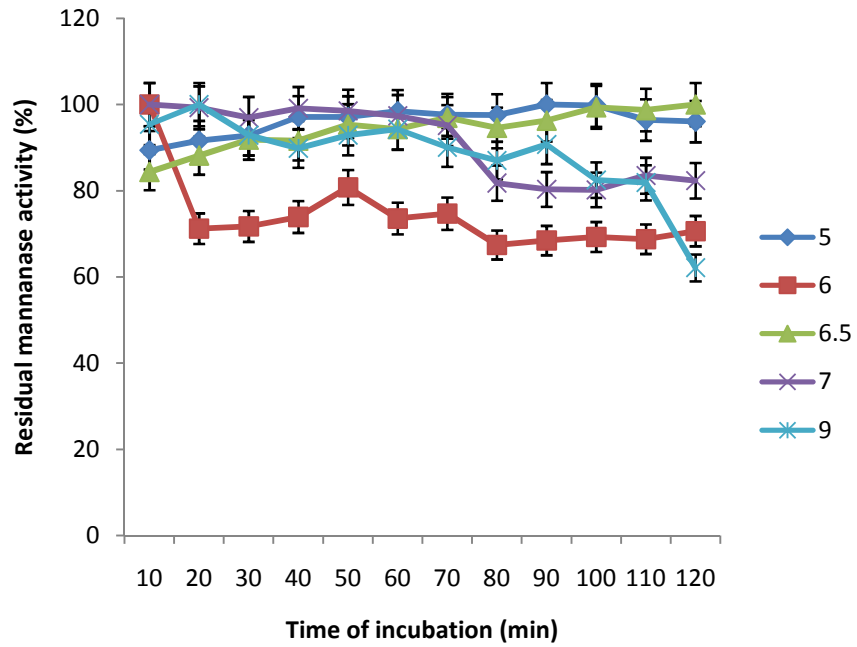


Fig. 6. Effect of pH on  $\beta$ -mannanase stability

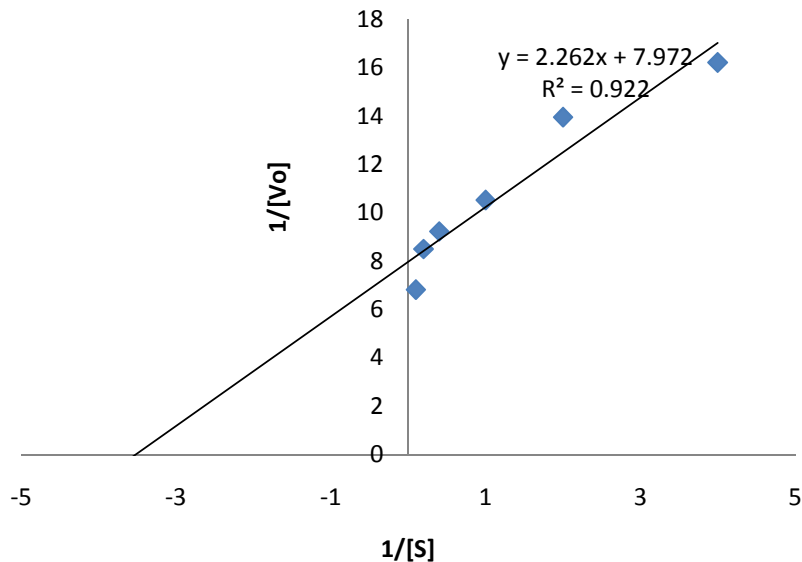


Fig. 7. Line weaver-Bulk double reciprocal plot ( $K_m=0.29$  mg/mL,  $V_{max}=0.13$   $\mu\text{mol}/\text{min}/\text{mL}$ )

Table 2. Effect of different salts and organic compounds on  $\beta$ -mannanase activity

Salts	Residual activity (%)			
	10mM	20mM	40mM	60mM
Control	100	100	100	100
MgCl <sub>2</sub>	0	0	0	0
NaCl	17.46	20	33.77	15.4
CuSO <sub>4</sub>	88.30	41.74	52.91	36.78
KCl	7.7	27.25	32.08	0.20
CaCl <sub>2</sub>	0	66.38	68.21	121.21
EDTA	50.03	41.45	2.44	0.56
FeSO <sub>4</sub> .7H <sub>2</sub> O	68.74	198.07	126.02	116.89
SDS	61.98	108.86	56.73	55.35
ZnSO <sub>4</sub> .7H <sub>2</sub> O	24.14	103.77	24.01	21.46
H <sub>2</sub> O <sub>2</sub>	12.63	26.09	2.25	15.45
BaCl <sub>2</sub>	0	18.84	68.49	57.22

Generally, enzyme activities almost dependent on changes with the pH of the reaction mixture which not only influence the enzyme activity, but also affect the  $K_m$  and  $V_{max}$  [31]. In this study, the optimum pH for  $\beta$ -mannanase was 5.0. The report is similar to those obtained for  $\beta$ -mannanase from other sources. Optimum pH of 5.5 was reported by Abdel-Fattah et al. [32] for *Aspergillus oryzae* NRRL 3448, while [24] reported pH optimum of 5.0 for *Scopulariopsis candida*. Optimum pH that falls between 6.0 to 7.0 for *Geobacillus stearothermophilus* L-07 was reported by [22] at different stages of purification. The fact that the  $\beta$ -mannanase is active over a wide pH range may imply that the enzyme will be useful in processes that are subjected to wide pH changes from acidic to alkaline range and vice-versa. Decline in reaction rates or enzyme activities at high or low pH values may reflect enzyme denaturation or dissociation of essential cofactor [27].

The concentrations of salts and organic compounds used in this study exerted their effect on  $\beta$ -mannanase activity. In this investigation, specific concentrations of salts and organic compounds were stimulatory, while others were inhibitory to enzyme activity. The activities of  $\beta$ -mannanase varied greatly with the concentrations of salts and organic compounds; however, the degree of inhibition was obvious in almost all the concentrations of salts and organic compounds. The stimulation of enzyme activities by  $\text{Fe}^{3+}$  and  $\text{Ca}^{2+}$  has been documented [5,33]. In this case, metal ions from these salts may form an integral part of the active enzyme or it may combine with the substrate to give the true substrate of enzyme [32]. Notably, the concentrations of EDTA,  $\text{BaCl}_2$  and  $\text{H}_2\text{O}_2$  employed in this investigation were inhibitory to the activity of  $\beta$ -mannanase produced by *P. italicum*. It was suggested by [34] that EDTA acted by chelating  $\text{Ca}^{2+}$  with a resultant loss of catalytic activity of the enzyme. The inactivation of enzyme via heavy metal poisoning has been well documented [33]. They are known to react with protein sulphhydryl groups, thus converting them to mercaptides. These reactions could lead to alteration of the enzyme protein, thus render it less soluble and frequent precipitation.

The apparent  $K_m$  value for LBG hydrolysis fall within the values reported by Meenakshi et al. [5] and Abdel-Fattah et al. [32]. The  $K_m$  indicated the concentration of substrate to fill the half active sites of an enzyme. It is also a measure of strength of the enzyme-substrate (ES) complex. In fact, [34,35] reported that a high  $K_m$  value indicates weak binding and vice versa. Therefore, this  $\beta$ -mannanase with low  $K_m$  value has strong affinity for the substrates.

## 5. CONCLUSION

The result obtained from this study revealed that purified  $\beta$ -mannanase is active over a wide pH and temperature, and its stability implies that the enzyme will be useful in processes that are subjected to extreme conditions. Therefore, this enzyme can withstand some harsh conditions encountered during industrial operations. The low  $K_m$  value obtained suggests that this purified enzyme has high affinity toward substrates and can possibly be used for prebiotic and animal feed formulation.

## COMPETING INTEREST

Authors have declared that no competing interests exist.

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